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Microgeographic Population Genetic Structure of the Mummichog (Fundulus heteroclitus L) Inhabiting an Industrialized Waterway

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Microgeographic Population Genetic Structure of the Mummichog (*Fundulus heteroclitus* L.) Inhabiting an Industrialized Waterway

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Master of Science

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by

Luis Felipe Arzayus

2000

This thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Science

Luis Felipe Arzayus

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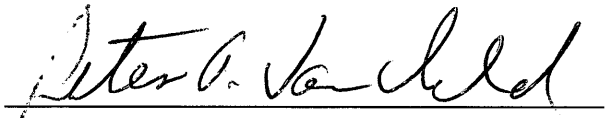

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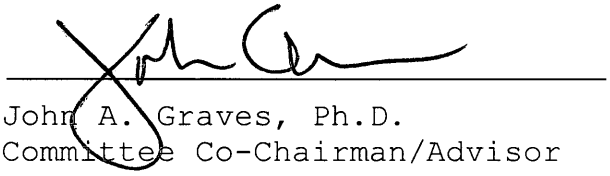

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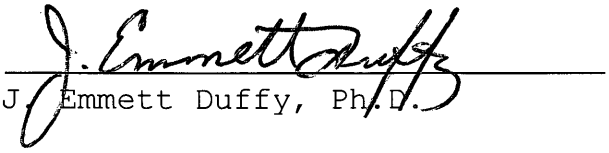

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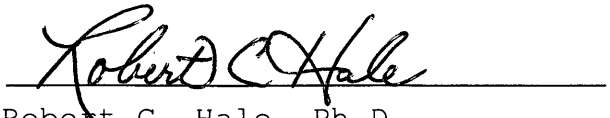

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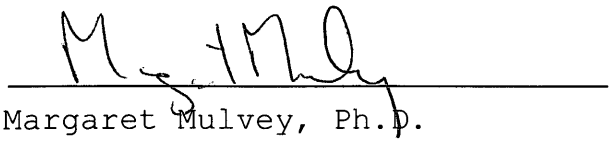

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TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS v

LIST OF TABLES vi

LIST OF FIGURES vii

LIST OF APPENDICES viii

ABSTRACT ix

INTRODUCTION 1

Population Structure of *Fundulus heteroclitus* 1
Study Overview 4

MATERIALS AND METHODS 9

Sampling Sites and Fish Collection 9
Molecular Markers 17
Allozyme Electrophoresis 17
Restriction Fragment Length Polymorphism (RFLP) Analysis of Whole Molecule Mitochondrial DNA 19
RFLP Analysis of the Mitochondrial D-loop and ND-4 Regions 21
Statistical Analyses 23
Allozyme Data 23
Mitochondrial Data 25

RESULTS 27

Allozyme Analysis 27
Comparisons Between the York River Site and the Combined Elizabeth River Sites 33
Comparison Among Elizabeth River Sites 35
Allozyme Data Summary 41
Mitochondrial Restriction Fragment Length Polymorphism (RFLP) Analysis 44
Whole Molecule Mitochondrial DNA RFLP Data 44
Mitochondrial D-loop and ND-4 Restriction Fragment Data 45
Comparison Between the York River Site and the Combined Elizabeth River Sites 55
Comparison Among Elizabeth River Sites 56
Mitochondrial Data Summary 63

DISCUSSION 64

Temporal Heterogeneity 66

Geographic Population Structure of *Fundulus heteroclitus* 67
Population Structure of *Fundulus heteroclitus* from the Elizabeth and York rivers 68
CONCLUSIONS 73
Future Research 75
APPENDICES 76
LITERATURE CITED 88
VITA 95

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LIST OF TABLES

Table		Page
1.	Total PAH concentrations reported for the Elizabeth and York river collection sites . . .	10
2.	Geographic distance among Elizabeth River sites	11
3.	Thermo-cycler protocols	22
4.	Preliminary screening of enzyme systems	28
5.	Allele frequencies for eight polymorphic allozyme loci	30
6.	Allozyme genetic variation	34
7.	Chi-square tests for deviation from Hardy-Weinberg equilibrium	37
8.	Contingency chi-square analysis of genetic heterogeneity	39
9.	Wright's statistics for three hierarchical levels of population structure.	40
10.	Distribution of significant allele heterogeneity between and among locations	48
11.	Estimated size (bp) of mitochondrial DNA (mtDNA) D-loop and ND-4 restriction fragments from digests with 12 enzymes	51
12.	Composite mtDNA haplotypes from 12 restriction enzymes	54
13.	Summary mtDNA statistics (D-loop and ND-4 regions).	60
14.	Net mean nucleotide sequence divergences between Elizabeth and York river samples	61
15.	Evaluation of chi-square homogeneity using Monte Carlo simulations	62

LIST OF FIGURES

Figure		Page
1.	Map of the Elizabeth River	12
2.	Map of the York River.	15
3.	Nei's (1972) genetic distance estimated from eight polymorphic loci	41
4.	Mantel correlation between Nei's (1978) genetic distance and geographic distance	46
5.	Whole mtDNA restriction fragment profiles and composite haplotypes	49
6.	Relationship among *Fundulus heteroclitus* mtDNA haplotypes	57

LIST OF APPENDICES

Appendix		Page
1.	Preliminary screening of polymorphic restriction enzymes for the mitochondrial D-loop and ND-4 regions	76
2.	Allozyme summary for 1997 and 1998 collections	80
3.	Estimates of evolutionary distance derived from restriction site data of 31 composite haplotypes.	86

ABSTRACT

Analysis of the microgeographic population genetic structure of the mummichog *Fundulus heteroclitus* was undertaken to evaluate the possibility of genetic differentiation between individuals inhabiting polluted and pristine sites. Samples of approximately 100 individuals each were collected in 1997 and 1998 from five locations within the southern branch of the Elizabeth River VA (ER), a highly industrialized sub-estuary, and a pristine location on the York River, VA. Samples were analyzed for genetic variation at eight polymorphic allozyme loci and restriction fragment length polymorphisms of the mitochondrial DNA control (D-loop) and nicotinamide dehydrogenase-4 (ND-4) regions. Variation was high within the overall collection, with a mean heterozygosity for the eight polymorphic allozyme loci of $\overline{H}$ = 0.201, and a nucleon diversity of the combined mitochondrial gene regions of h = 0.366. Comparisons of samples taken from the same location in consecutive years revealed no significant temporal heterogeneity at any location for any of the variable molecular markers. Samples taken in different years were pooled for subsequent analyses of spatial heterogeneity. Comparison of samples from the Elizabeth and York rivers demonstrated significant heterogeneity at one allozyme locus and in the distribution of mtDNA composite haplotypes. Similar levels of heterogeneity were found among five of ten samples within the Elizabeth River. Three of five allozyme loci were heterogeneous among ER samples as well as five pair-wise comparisons of mtDNA composite haplotype distributions. In all cases, significant heterogeneity values were attributed to deviant allele frequencies for one locus at one collection location, and there was no consistent pattern (by locus or location) to these deviations. Mummichog from highly polluted sites were no more genetically distinct than those from other locations. The results of this study suggest there is sufficient gene flow among *F. heteroclitus* to maintain genetic similarity over large areas, but factors such as the size of local effective populations and differential reproductive success within samples may result in significant heterogeneity on a microgeographic scale.

INTRODUCTION

The mummichog (*Fundulus heteroclitus*) occurs in shallow estuaries and tidal creeks along the east coast of North America, from Nova Scotia (Canada) to northern Florida (Atz 1986). *Fundulus heteroclitus* exhibits marked sexual dimorphism and a polygynous mating system (Atz 1986). Mark-recapture studies in a tidal creek estimated the home range of *F. heteroclitus* to be approximately 40 m during summer and 2000 m during winter seasons (Fritz et al. 1975; Lotrich 1975). Non-planktonic eggs limit the potential for dispersal of early life history stages (Kneib 1986). Following air incubation after spring tidal immersion, larvae and juveniles remain near the shallow pools where they hatched (Able and Felley 1986; Morin and Able 1983). These life history traits limit gene flow, making *F. heteroclitus* an excellent marine fish to evaluate the effects of evolutionary processes that form and maintain population structure on a microgeographic scale.

Population Structure of *Fundulus heteroclitus*

Fundulus heteroclitus exhibits considerable population structure. Physiological, behavioral, morphological and genetic differences have been demonstrated for 'northern'

type *F. heteroclitus* inhabiting the upper portions of Chesapeake and Delaware bays and coastal areas from northern Maryland to Nova Scotia (Canada), and 'southern' type *F. heteroclitus* inhabiting lower portions of Chesapeake and Delaware bays and coastal waters from Virginia to northern Florida (Powers and Schulte 1998). Latitudinal changes in mean water temperature along the east coast of North America may explain some physiological and behavioral variations in *F. heteroclitus*. For instance, the kinetic properties of the heart type lactate dehydrogenase enzyme and *F. heteroclitus* swimming endurance were significantly correlated with temperature (DiMichele and Powers 1982).

Northern and southern forms of *F. heteroclitus* also exhibit different reproductive behaviors and egg architectures, with a break in these characters in Long Island, New York (Able and Felley 1986). Northern types deposit eggs in algal mats or on sandy beaches, while southern types deposit eggs in empty shells and between leaves of the seagrass *Spartina alterniflora* (Halpin 1997; Yozzo et al. 1994).

Genetic investigations of *F. heteroclitus* have revealed high levels of variation and considerable population structure. Allozyme analyses of *F. heteroclitus*

indicated significant clinal variation (i.e., directional change in allele frequencies as a function of geography) for several presumptive gene loci (Cashon et al. 1981; Powers and Place 1978; Ropson et al. 1990). Two general models can explain cline formation and maintenance: primary and secondary contact (Endler 1977). In the primary contact model, natural selection produces changes in allele frequencies over an environmental gradient. Over time, the result is a cline in allele frequencies. In the secondary contact model, populations are initially isolated as a result of some barrier to gene flow. Genetic drift of neutral alleles, and/or adaptive forces, result in allelic differences between the disjunct populations. Once the barrier is removed and gene flow (exchange) reestablished, a cline in allele frequencies results.

To distinguish between the primary and secondary contact models, Gonzalez-Villaseñor and Powers (1990) investigated the spatial distribution of mitochondrial (mtDNA) restriction site variation in *F. heteroclitus*. Two distinct mtDNA lineages were revealed with a sharp transition zone between lineages located in the area between 39° 40' and 39° 42' N latitude (Gonzalez-Villaseñor and Powers 1990). Thus, the clinal variation shown by some allozyme loci, as well as some morphological features, is

consistent with secondary contact (Able and Felley 1986; Gonzalez-Villaseñor and Powers 1990), although the effects of selection cannot be ruled out.

Few studies have characterized the population structure of *F. heteroclitus* on smaller spatial scales. Weis and Weis (1989) reported significant allozyme and morphological variation between *F. heteroclitus* occurring in a site contaminated by methyl mercury (meHg) and fish from a pristine site approximately 34 km apart. Mitton and Koehn (1975) reported significant heterogeneity of allele frequencies for some allozyme loci between samples of *F. heteroclitus* inhabiting a power plant effluent and those inhabiting several control sites within a 10 km area of Long Island Sound. An analysis of mtDNA restriction site variation of five samples of *F. heteroclitus* collected from locations along a spatially homogeneous area (930 - 8000 m) showed no evidence of geographic structuring or genetic differentiation (Brown and Chapman 1991).

Study Overview

The southern branch of the Elizabeth River, Virginia (USA), a small subestuary of the Chesapeake Bay, is highly industrialized with large shipyards, coal loading docks, and several abandoned wood treatment facilities, and a fuel

refinery (Figure 1). In addition, this waterway is continuously dredged to facilitate commercial shipping and shipyard accessibility (Huggett et al. 1992). During the past century, the southern branch of the Elizabeth River has been subjected to several massive chemical spills as well as chronic non-point inputs of creosote, a common wood preservative (Bieri et al. 1986). Creosote is composed of a mixture of polycyclic aromatic hydrocarbons (PAHs) and phenolic and heterocyclic compounds (Hale and Aneiro 1997; Kiilerich and Arvin 1996). One abandoned wood treatment facility, formerly known as Atlantic Wood Industries, is one of the most heavily PAH contaminated estuarine habitats in the United States and is currently designated a Superfund site (USEPA 1999; van der Leeden 1991).

Vogelbein et al. (1990) reported high prevalence of hepatic neoplasms, as well as a broad spectrum of preneoplastic and non-neoplastic liver lesions within *F. heteroclitus* collected at the Atlantic Wood (AW) site. Significantly lower frequencies of hepatic lesions were found in fish collected from Scuffletown Creek (SC), an area located 600 m across the river channel from AW with PAH concentrations three orders of magnitude lower than those at AW. Williams (1994) investigated the possibility that *F. heteroclitus* at AW were tolerant of PAH

contaminated sediments. *Fundulus heteroclitus* collected from AW and the Catlett Islands (CI), a pristine site located in the York River, were bred in the laboratory. F_1 individuals from AW and CI parents were exposed to creosote amended sediments (Williams 1994). After 7 days of exposure, progeny from crosses AW X AW adults had 25% mortality while progeny from crosses CI X CI adults exhibited 100% mortality. F_1 progeny of female AW X male CI crosses exhibited significantly lower mortality (97%) than CI X CI progeny ($P = 0.04$). Since crosses using male AW X female CI did not produce offspring with significantly lower mortalities than CI, Williams suggested that resistance to creosote may be maternally mediated. Williams (1994) inferred that fish from AW were resistant to creosote and that this resistance was heritable and may have a genetic basis.

The present study follows from Williams' (1994) observations and was undertaken to characterize the microgeographic genetic structure of *F. heteroclitus* inhabiting the heavily polluted AW location, as well as other less polluted areas in the Elizabeth River. *Fundulus heteroclitus* from the Catlett islands (CI), a pristine location on the York River, were also included as part of the reference sample. Analysis of the microgeographic

genetic population structure of *F. heteroclitus* was used to evaluate the possibility of genetic divergence between mummichog inhabiting polluted and pristine sites. The following questions were asked:

1. Do patterns of genetic variation differ significantly between *F. heteroclitus* collected from sites in the industrialized southern branch of the Elizabeth River VA and a pristine site in the York River VA?
2. Are patterns of genetic variation significantly different among *F. heteroclitus* collected from sites within the Elizabeth River, VA with varying PAH-sediment concentrations?

The objectives of this research were addressed by surveying three classes of molecular markers incorporating two modes of inheritance. Allozymes were used as markers of the nuclear genome, which is biparentally inherited and undergoes recombination. Several polymorphic allozyme loci have been characterized in southern Chesapeake Bay *F. heteroclitus* (Cashon et al. 1981; Place and Powers 1978; Powers et al. 1986; Ropson et al. 1990). The non-recombining, uniparentally transmitted mitochondrial genome

was surveyed with two techniques. Restriction fragment length polymorphism (RFLP) analysis of whole molecule mtDNA was used to confirm that individuals from a sample site were the southern form of *F. heteroclitus* previously described in the literature. Polymerase chain reaction (PCR) amplified mitochondrial DNA encompassing the hypervariable control region (D-loop) and the more conservative nicotinamide dehydrogenase-4 (ND-4) region were also assayed for RFLPs to survey genetic diversity of *F. heteroclitus*.

MATERIALS AND METHODS

Sampling Sites and Fish Collection

Fundulus heteroclitus were collected during the summers of 1997 and 1998 from five locations in the southern branch of the Elizabeth River, Virginia (USA), and from a reference site in the York River, Virginia (USA). Approximately 100 fish were collected from each site each year. Sediment creosote concentrations at collection sites were reported to vary over a wide range, from near pristine conditions to total PAH concentrations as high as 133,000 mg/kg (Table 1).

Collection sites were selected within a small geographical area along the Elizabeth River on both sides of the river channel (Figure 1 and Table 2). Atlantic Wood (AW) and Paradise Creek (PC) are situated on the west bank of the Elizabeth River channel. The AW collection site is located in a shallow, tidal creek near a former wood treatment facility. Raw creosote, which was used as a wood preservative, was known to spill during impregnation and run off from this facility into the collection site. The AW site has been extensively studied by investigators from the Virginia Institute of Marine Science (VIMS) and is well characterized. The PC collection site is located on a

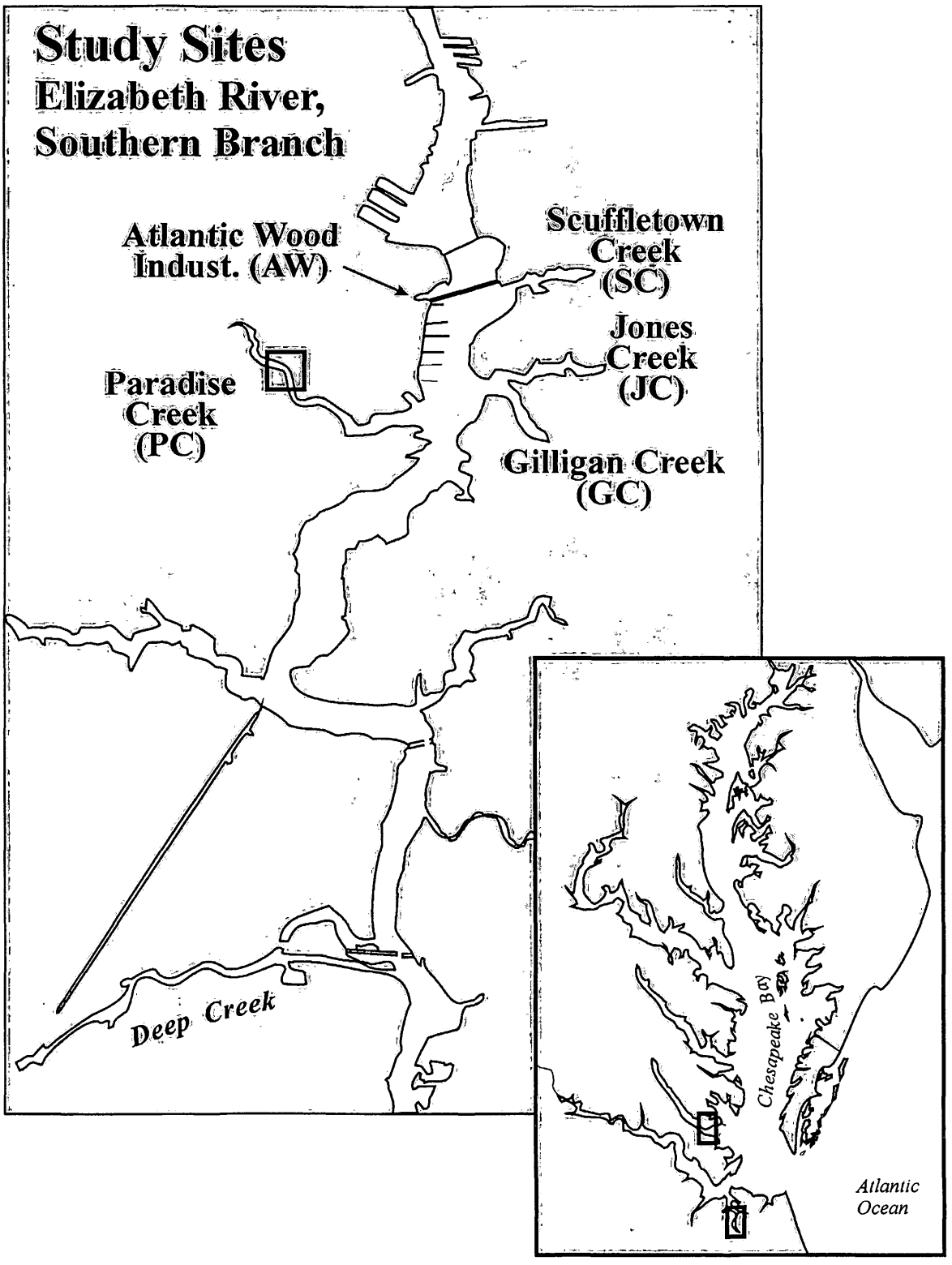
Table 1. Total sediment PAH concentrations reported for collection sites on the Elizabeth and York rivers. * Indicates samples collected at the mouth of the creek on the Elizabeth River.

Location	Σ PAH (mg/kg dry sediment Vogelbein et al. (1990)	Vogelbein et al. (1993)
Elizabeth River		
Atlantic Wood (AW)	2,200	133,000
Scuffletown Creek (SC)	61	88
Jones Creek (JC)		134*
Gilligan Creek (GC)		134*
Paradise Creek (PC)		83
York River		
Catlett Islands (CI)		0.30

Table 2. Distance in meters between collection sites in the Elizabeth River. Distance was measured 'over water' to reflect swimming distance (see map in Figure 1).

	Atlantic Wood	Scuffletown Creek	Gilligan Creek	Jones Creek	Paradise Creek
Atlantic Wood	-	868	1783	2011	2788
Scuffletown Creek			1691	1920	3017
Gilligan Creek				1120	2377
Jones Creek					2606
Paradise Creek					-

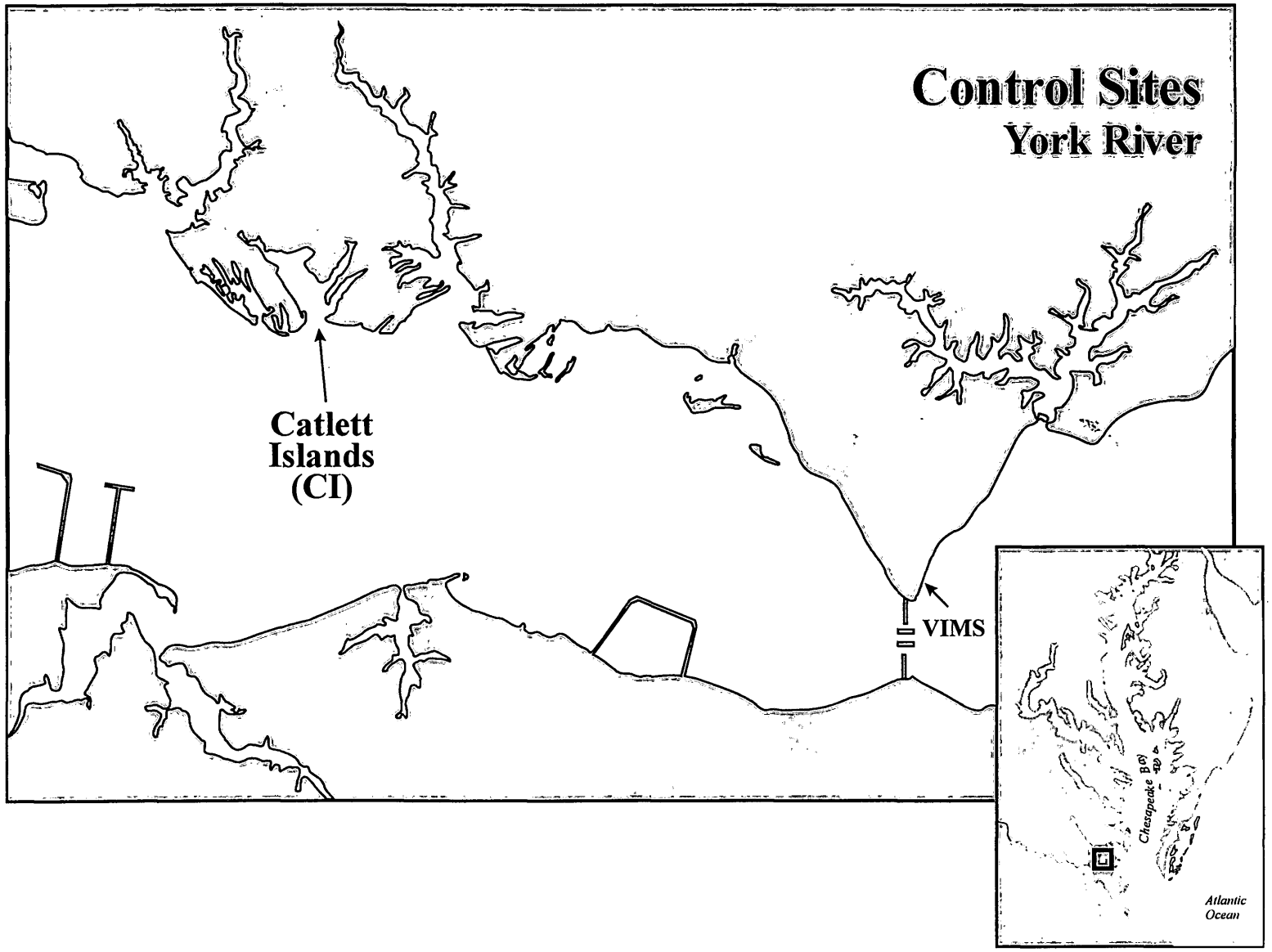
Figure 1. Location of collection sites on the southern branch of the Elizabeth River, Virginia. Elizabeth and York rivers in the Chesapeake Bay are shown in lower box.


Study Sites
Elizabeth River,
Southern Branch
Atlantic Wood
Indust. (AW)
Scuffletown
Creek
(SC)
Jones
Creek
(JC)
Paradise
Creek
(PC)
Gilligan Creek
(GC)
Deep Creek
Chesapeake Bay
Atlantic
Ocean

Creek 850 m west off the Elizabeth River channel. Scuffletown Creek (SC), Jones Creek (JC) and Gilligan Creek (GC) are located on the eastern bank of the channel. The SC site is located in a tidal creek across from the AW site. The GC and JC collection sites are located in two creeks south of SC, 680 m off the main channel. The GC and JC sites are characterized by thick vegetation (*Spartina* species); prominent features at the mouth of these two sites include a barge loading facility for coal and fertilizer products and an oil tank farm on the southeast end. In the York River, the Catlett Islands (CI) site was used as a reference (non-industrialized) collection location. The area is designated as a NOAA National Estuarine Research Reserve System (NEERS) site (Figure 2). Previous research characterized the total PAH concentration in the CI area as minimal (Vogelbein et al. 1990)(Table 2).

Fundulus heteroclitus were caught using Gee-minnow traps (Cuba Specialty Manufacturing, Fillmore, NY) baited with crabmeat. Traps were set near the vegetation line in water approximately 60 - 80 cm deep one hour prior to the predicted time of high tide. Traps were left immersed for one hour. After retrieving the traps, fish were placed in a 40-L cooler filled with 2-cm of continuously aerated ambient water. Approximately 100 adult fish, male and

Figure 2. Location of the reference site, Catlett Islands (CI), on the York River, Virginia. VIMS: Virginia Institute of Marine Science.


Control Sites
York River
Catlett
Islands
(CI)
VIMS
Chesapeake Bay
Atlantic
Ocean

female, (mean weight 5.96 g; mean length 89.8 mm) were culled indiscriminately from the cooler and kept for analysis. Remaining fish were returned to the same location from which they were collected. At VIMS, the fish were kept alive until processing in a 300-L flow-through tank receiving sand and carbon filtered York River water. During this time, *F. heteroclitus* were fed *ad libitum* Tetramarin© commercial marine food.

Molecular Markers

Allozyme Electrophoresis

Each *Fundulus heteroclitus* was assigned a number and sacrificed by immersion in a 500 mg/L solution of tricaine methanesulphonate (MS-222) (Argent Chemical Co.). Tissues were dissected and processed as described by Place and Powers (1978) with the following modifications. A homogenate was prepared separately from liver, eye, and white muscle tissues by grinding each with a mechanical tissue homogenizer (Brinkmann Inc.) in 3.0 mL, 1.0 mL and 0.1 mL, respectively, of chilled grinding buffer. The resulting homogenate was centrifuged at 20,000*g*, 4 °C for 30 minutes and the supernatant decanted into standard 1.5 mL micro-centrifuge tubes. Homogenates were stored in an ultracold (-80°C) freezer.

Preliminarily, homogenates from thirty *F. heteroclitus* collected at a site on the York River were screened for twenty-two enzymes systems. These enzyme systems were selected based on reported polymorphisms within *F.* heteroclitus from the southern Chesapeake Bay and the availability of stains in the laboratory.

Homogenates were subjected to electrophoresis on 12% (w/v) horizontal starch (Starch Art Inc, TX) gels using the wickless gel mold and apparatus described in Murphy et al. (1996). Proteins were separated electrophoretically in a continuous Tris-citrate II buffer (Selander et al. 1969) run for 12 - 16 hr at 3.8 V/cm using a constant voltage power supply.

Differences in migration for each enzyme were visualized through histochemical staining. Sets of band patterns specific to each enzyme were first identified in relation to tissue source and known quaternary structure. Each set of bands particular to an enzyme and tissue were then assigned a locus designation (i.e., LDH-A) in accordance to the standardized set of systematic and trivial names for enzymes compiled by the Enzyme Commission (E.C.) of 1973 (Richardson et al. 1986). Alleles for each locus were designated according to relative migration distance (i.e., 'F' for fast; 'M' for medium). The

combination of the two alleles typical of diploid fish, form the fish's genotype (i.e., 'FM', 'FF' or 'MM'), which was used for scoring on the data sheets. After scoring, each gel slice was photographed with a digital gel documentation system. Allele designations were converted to numeric values in a spreadsheet in order of decreasing anodal mobility, with the most anodal allele being designated "1".

Restriction Fragment Length Polymorphism (RFLP) Analysis of Whole Molecule Mitochondrial DNA

Mitochondrial DNA (mtDNA) enriched genomic DNA was purified from liver *F. heteroclitus* tissue following the protocol of Chapman and Powers (1984). Aliquots of genomic DNA (5 to 20 ng) were digested with the following restriction endonucleases according to the directions of the manufacturer (Gibco/BRL): *Hin*d III, *Sma* I and, *Sst* I. These enzymes were among several reported to distinguish northern and southern forms of *F. heteroclitus* (Gonzalez-Villaseñor and Powers 1990; Smith et al. 1998). Following overnight digestion, restriction fragments as well as a biotinylated size standard (phage lambda DNA/*Hin*dII fragments; Gibco/BRL) and dilute probe (positive control), were separated on a 1.0% agarose gels. Gels were run on a

Gibco/BRL model H4 horizontal gel apparatus at 110 volts for 4 - 5 hours. Following electrophoresis gels were transferred to a 200 cm^2 nylon filter membrane via capillary action (Southern transfer, Sambrook et al. 1989) and immobilized by long-wave UV irradiation.

Bluegill (*Lepomis macrochirus*) mtDNA (~ 1 µg) was purified by cesium chloride density gradient ultracentrifugation according to the protocols of Lansman et al. (1981) and used as a heterospecific probe for *F. heteroclitus* mtDNA fragments. Bluegill mtDNA probe was nick-translated with the BioNick DNA labeling system following the protocols of the manufacturer (Gibco/BRL). Nylon membranes were prehybridized for two hours at 42°C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.025 mM $NaPO_4$, pH 6.5, and 100 µg/mL heat denaturated calf thymus DNA. One µg of labeled probe was added to the prehybridization solution and the filter was incubated overnight at 42°C. Hybridization membranes were then washed as described by the manufacturer's instructions (BioNick DNA labeling system; Gibco/BRL). MtDNA fragments were detected with the BlueGene Non-Radioactive Nucleic Acid Detection Kit (Gibco/BRL) according to the manufacturer's instructions.

Fragment lengths were estimated against a known size standard (biotinylated λ/*Hin*dIII; Gibco/BRL), and fragment patterns were recorded for each individual.

RFLP Analysis of the Mitochondrial D-loop and ND-4 Regions

Genomic DNA was isolated from liver tissue following the protocol of Winnepennickx (1993) modified for teleost fishes. The mtDNA D-loop gene region was amplified from DNA isolations by the polymerase chain reaction (PCR) using the universal primers of Martin et al. (1992). The ND-4 gene region was amplified using the primers of Bielawski and Gold (1996; Personal Communication) (Table 3). PCR amplifications were performed in a DNA Engine model PTC-200 thermal-cycler (MJ Research). Denaturing, annealing and extension temperatures and times are listed in Table 3. PCR products were digested overnight with a suite of restriction endonucleases used according to the manufacturer's (Gibco/BRL) directions (Appendix 1). After digestion, the resulting fragments were separated on 2.5% horizontal agarose gels consisting of 1.25% UltraPure agarose (Gibco/BRL) and 1.25% NuSeive GTG agarose (FMC BioProducts). Agarose gels were run on a Gibco/BRL model H4 horizontal gel apparatus at 100 volts for 3 - 4 hours. Gels were stained by immersion in 500 ml TBE and 20 µl of 5

Table 3. Thermocycler protocols for PCR amplification of *Fundulus heteroclitus* D-loop and ND-4 regions of the mitochondrial genome.

Steps	D-loop		ND4	
1. Initial denaturation	95 °C	5 min	95 °C	5 min
2. Denaturation	94 °C	1 min	94 °C	1 min
3. Annealing	45 °C	1 min	50 °C	1 min
4. Extension	65 °C	1 min	65 °C	3 min
5. Repeat 2-4 35x				
6. Final Extension	65 °C	7 min	65 °C	7 min

Primer sequences:

D-loop: (F) CAT ATT AAA CCC GAA TGA TAT TT
(R) ATA ATA GGG TAT CTA ATC CTA GTT T

ND-4: (F) CAA GAC CCT TGA TTT CGG CTC A
(R) CAA GAC TTT CAG GCT AAG ACC A

mg/mL ethidium bromide. Restriction fragments were visualized by UV transillumination. Fragment sizes were estimated by comparison to a known size standard (1KB DNA ladder or 123 base pair ladder; Gibco/BRL).

A total of thirty-four restriction enzymes were used in a preliminary screening of the PCR-amplified D-loop and ND-4 mitochondrial regions for 20 *F. heteroclitus* collected from a York River site (Appendix 1). The criteria used for the selection of a restriction enzyme was based on whether it produced polymorphic fragment patterns among the individuals, and if the sizes of the resulting fragments were completely additive to the uncut amplified region.

Composite haplotypes were formed by assigning letters indicating the fragment pattern produced by each restriction enzyme for each individual. The number of restriction site changes (gains or losses) between composite haplotypes was inferred from completely additive changes in fragment sizes.

Statistical Analyses

Allozyme Data

Allele frequencies, average heterozygosity, conformance of genotype frequencies to Hardy-Weinberg equilibrium expectations, Wright's F statistics, Nei's

(1978) and Roger's (1972) genetic identities and unbiased distance indices were determined with the computer program Biosys-2 (Swofford and Selander 1984).

Contingency chi-square statistics (Richardson et al. 1986) were used to assess temporal heterogeneity between *F. heteroclitus* samples collected in 1997 and 1998. For each collection site, allele frequencies from each locus were paired by collection year, and added to columns and rows to determine expected genotype frequencies. Observed (*O*) and expected (*E*) genotype frequencies were then analyzed to determine chi-squared probabilities as shown:

$$E = \frac{CxR}{GT} \Rightarrow \sum_{i=G} \frac{(O-E)^2}{E} = \chi^2$$

Where C = column, R = row and GT = total from C+R for each locus.

To avoid artificially inflated chi-square results when expected genotype frequencies were too small, genotypes were pooled into classes with expected values of greater than five individuals. If this resulted in a single class, the test was excluded (Richardson et al. 1986). The sequential Bonferroni method (Rice 1989) was used to correct for the probability of encountering false significant values resulting from multiple tests.

Mitochondrial Data

A composite mtDNA haplotype representing the restriction fragment patterns of each enzyme was developed for each *F. heteroclitus*. Haplotype diversity (*h*), a measure of the variation within a population, was estimated for each sample and for the pooled samples following Nei (1987). Nucleotide sequence divergence between haplotypes (*d*) was estimated following Nei and Miller (1990), and the divergences between samples were corrected for within-sample diversities (δ_{net}). The homogeneity of haplotype distributions among samples was evaluated by chi-square analyses using the Monte-Carlo randomization protocol of Roff and Bentzen (1989) with 1000 randomizations of the data. The above were calculated using version 4.0 of the Restriction Enzyme Analysis Package (REAP) of McElroy et al. (1991). A hierarchical analysis of variance (Schneider et al. 1997), was used to partition the total variance into intra-and inter population components without considering allele relationships (θ). These components were then used to estimate fixation indices (F_{ST}) and inbreeding coefficients (F_I) among sites and between rivers (Weir and Cockerham 1984).

A matrix correlation (Mantel test)(Sokal 1979) was carried out between Rogers' (1972) and Nei's (1978) genetic

distances and geographic distance between collection sites (nuclear data). Net nucleotide sequence divergence (mitochondrial data) and geographic distance were tested similarly using the spatial autocorrelation analysis program of Wartenberg (1989).

RESULTS

Allozyme Analysis

In a preliminary survey, 30 *Fundulus heteroclitus* collected at a York River site were screened for 22 enzyme systems (Table 4). Eight enzymes (36%) were not resolved consistently (EST, G3PDH, PEP-*leu-gly-gly*, PEP-*leu-pro*, ADH, PER, MDHP and ACOH); six enzymes (28%) were monomorphic (GP, PGDH, PK, SOD, G6PDH, and GCDH); and eight enzymes (36%) were polymorphic (AAT, FUM, GPI, IDH, LDH, MDH, MPI and PGM). All samples (n = 582) were subsequently scored for those loci that were resolved consistently and found to be polymorphic in the preliminary screening. Allele frequencies for the eight loci are presented in Table 5 and each locus is discussed below.

Lactate dehydrogenase (LDH), a tetrameric protein, revealed three loci in eye tissue, and two loci in liver and white muscle tissues. The most anodally migrating band (*Ldh*-C) was specific to eye tissue and non-variable. The least anodally migrating band (*Ldh*-B), which was specific to liver tissue, had three alleles and was surveyed in all samples. The frequency for the most common allele was 0.79. The second most anodally migrating band, particular

Table 4. Enzyme systems examined in a preliminary allozyme screening of *Fundulus heteroclitus*.

Enzyme Abbreviation	Enzyme name	Standardized nomenclature (E.C)
AAT	Aspartate aminotransferase	EC 2.6.1.1
ACOH	Aconitate hydratase	EC 4.2.1.3
ADH	Alcohol dehydrogenase	EC 1.1.1.1
EST	Esterase	EC 3.1.1.1
FUM	Fumarate hydratase	EC 4.2.1.2
G3PDH	Glycerol-3-phosphate dehydrogenase	EC 1.1.1.8
G6PDH	Glucose-6-phosphate dehydrogenase	EC 1.1.1.49
GCDH	Glucose dehydrogenase	EC 1.1.1.118
GP	General protein	Non-specific
GPI	Glucose-6-phosphate isomerase	EC 5.3.1.9
IDH	Isocitrate dehydrogenase	EC 1.1.1.42
LDH	Lactate dehydrogenase	EC 1.1.1.27
MDH	Malate dehydrogenase	EC 1.1.1.37
MDHP	Malate dehydrogenase (NADP+)	EC 1.1.1.40
MPI	Mannose-6-phosphate isomerase	EC 5.3.1.8
PEP-*leu-gly-gly*	Peptidase	EC 3.4.-.-
PEP-*leu-pro*	Peptidase	EC 3.4.-.-
PER	Peroxidase	EC 1.11.1.7
PGDH	Phosphogluconate dehydrogenase	EC 1.1.1.44
PGM	Phosphoglucomutase	EC 5.4.2.2
PK	Pyruvate kinase	EC 2.7.1.40
SOD	Superoxide dismutase	EC 1.15.1.1

to white muscle tissue, was not resolved consistently and thus not scored.

Isocitric dehydrogenase (IDH), a dimeric protein, had three well-resolved alleles when assayed in liver tissue (*Idh*-B). The frequency of the most common allele was 0.683. *Idh*-A was monoallelic.

Glucose-6-Phosphate isomerase (GPI), a dimeric protein, was easily scored from liver tissue. Two loci were resolved. The least anodally migrating locus (*Gpi*-A) had two alleles, and the frequency of the most common allele was 0.853. *Gpi*-B was also anodal but monoallelic.

Aspartate aminotransferase (AAT), a dimeric enzyme, revealed two loci: one migrated anodally while the other migrated cathodally. The anodal locus (*Aat*-1) was polymorphic and surveyed in this study. *Aat*-1 had three alleles; the frequency of the most common was 0.943. The cathodal locus (*Aat*-2) was not readable consistently and therefore not used.

Phosphoglucomutase (PGM), a monomeric enzyme, had four alleles when screened in white muscle tissue (*Pgm*-A). The frequency of the most common allele was 0.826.

One locus was resolved from white muscle tissue for mannose-6-phosphate isomerase (MPI), a monomeric enzyme.

Table 5. Allele frequencies of eight polymorphic allozyme loci at six collection sites of *F. heteroclitus* collected in 1997 and 1998. Alleles at each locus are numbered in order of decreasing anodal mobility; (n) = sample size.

1997		Atlantic Wood	Scuffle-town Creek	Gilligan Creek	Jones Creek	Paradise Creek	Cattlet Islands
Ldh-B	*n*	57	36	34	57	31	62
	1	-	0.069	-	-	-	0.008
	2	0.921	0.750	0.750	0.798	0.790	0.798
	3	0.079	0.181	0.250	0.202	0.210	0.194
Gpi-A	*n*	58	36	34	57	31	62
	1	0.233	0.139	0.088	0.298	0.258	0.040
	2	0.767	0.861	0.912	0.702	0.742	0.960
Idh-B	*n*	58	36	34	57	30	62
	1	-	0.056	0.044	0.018	-	0.056
	2	0.603	0.750	0.750	0.807	0.75	0.669
	3	0.397	0.194	0.206	0.175	0.25	0.274
Mdh-A	*n*	58	36	34	57	31	62
	1	0.017	-	0.015	-	-	-
	2	0.983	1.000	0.971	1.000	1.000	1.000
	3	-	-	0.015	-	-	-
Pgm-A	*n*	58	36	34	56	31	62
	1	-	0.014	-	-	-	-
	2	0.905	0.889	0.838	0.830	0.839	0.823
	3	0.026	0.056	0.118	0.143	0.065	0.024
	4	0.069	0.042	0.044	0.027	0.097	0.153
Fum-A	*n*	58	36	34	57	31	62
	1	0.017	0.069	0.015	0.035	0.016	0.048
	2	0.983	0.931	0.985	0.965	0.984	0.944
	3	-	-	-	-	-	0.008
Mpi-A	*n*	58	36	34	57	31	62
	1	-	-	-	0.009	0.016	-
	2	0.940	0.958	0.971	0.886	0.887	0.968
	3	-	0.028	-	0.070	-	0.016
	4	0.060	0.014	0.029	0.035	0.097	0.016
Aat-1	*n*	58	36	34	57	31	62
	1	0.026	-	-	0.026	0.145	0.097
	2	0.966	0.986	1.000	0.974	0.855	0.903
	3	0.009	0.014	-	-	-	-

1998		Atlantic Wood	Scuffle-town Creek	Gilligan Creek	Jones Creek	Paradise Creek	Cattlet Islands
Idh-B	*n*	52	49	62	46	46	50
	1	0.010	0.092	0.008	-	-	0.040
	2	0.885	0.724	0.710	0.815	0.815	0.720
	3	0.106	0.184	0.282	0.185	0.185	0.240
Gpi-A	*n*	52	49	62	48	48	50
	2	0.135	0.102	0.040	0.208	0.208	0.050
	3	0.865	0.898	0.960	0.792	0.792	0.950
Idh-B	*n*	52	49	62	46	46	50
	1	0.010	0.061	0.040	0.065	0.065	0.070
	2	0.587	0.724	0.613	0.717	0.717	0.600
	3	0.404	0.214	0.347	0.217	0.217	0.330
Mdh-A	*n*	52	52	62	48	48	50
	1	-	-	0.040	0.010	0.010	-
	2	1.000	1.000	0.960	0.990	0.990	1.000
	3	-	-	-	-	-	-
Pgm-A	*n*	52	52	62	46	46	50
	1	0.010	0.010	-	0.022	0.022	0.010
	2	0.798	0.856	0.742	0.848	0.848	0.730
	3	0.077	0.038	0.121	0.054	0.054	0.100
	4	0.115	0.096	0.137	0.076	0.076	0.160
Fum-A	*n*	52	49	62	48	48	50
	1	0.048	0.092	-	0.021	0.021	0.020
	2	0.952	0.908	1.000	0.979	0.979	0.980
	3	-	-	-	-	-	-
Mpi-A	*n*	52	52	62	48	48	50
	1	-	-	0.016	-	-	-
	2	0.885	0.952	0.911	0.969	0.969	0.970
	3	-	0.038	0.024	0.021	0.021	0.020
	4	0.115	0.010	0.048	0.010	0.010	0.010
Aat-1	*n*	52	52	62	48	48	50
	1	-	-	0.024	0.083	0.083	0.060
	2	0.990	1.000	0.960	0.896	0.896	0.880
	3	0.010	-	0.016	0.021	0.021	0.060

This locus (*Mpi*-A), was polymorphic with four alleles. The frequency for the most common allele was 0.983.

Two loci were resolved for malate dehydrogenase (MDH), a dimeric enzyme. The *Mdh*-A locus, particular to white muscle, had three alleles; one allele was predominant in all samples with a frequency of 0.991.

Fumarase (FUM), a tetrameric enzyme revealed one locus with two alleles when using white muscle tissue (*Fum*-A). The frequency of the most common allele was 0.966.

No significant differences were noted between genotypic distributions and those expected under Hardy-Weinberg equilibrium for the 1997 and 1998 collection samples. On a locus-by-locus basis, 31 and 35 each of 48 possible tests for conformance to Hardy-Weinberg expectations were excluded for the 1997 and 1998 years, respectively. One locus (*Fum*-A), revealed significant deviations from Hardy-Weinberg estimates for the 1997 collection year, while there were no significant deviations from Hardy-Weinberg expectations for the 1998 collection year.

Temporal heterogeneity between samples collected in 1997 and 1998 was evaluated for each collection location using contingency chi-square statistics. In all instances, no statistically significant differences in allele

frequencies were observed between the 1997 and 1998 collections for any locus at any location ($X^2 \leq 3.8$; $p \geq 0.05$). Since there were no significant differences between years at any location, the data were pooled. Results reported below, therefore, represent data pooled from both collection years.

Comparisons Between the York River Site and the Combined Elizabeth River Sites

Mean heterozygosity ($\overline{H}$) estimates for *F. heteroclitus* collected in 1997 and 1998 from sites on the Elizabeth River and the control site on the York River ranged from 0.171 to 0.214 (Table 6).

There were no significant differences between observed mean heterozygosies and those expected under Hardy-Weinberg equilibrium for the York River and combined Elizabeth River samples. Mean heterozygosity for the York River site (CI; $\overline{H}$= 0.214 S.E. 0.071) and the combined Elizabeth River sites (AW, SC, GC, JC and PC; $\overline{H}$= 0.188 S.E 0.053) did not differ significantly (Table 6). The *Mdh*-A locus was monomorphic in CI resulting in a lower mean number of alleles per locus and number of polymorphic loci (2.8 and 7, respectively). On a locus-by-locus basis, after excluding seven tests with

Table 6. Genetic variation at all allozyme loci from all collection sites, data pooled from samples collected in 1997 and 1998. Heterozygosity based on random mating expectations (Nei, 1978). Standard errors are in parentheses.

Rivers / Sites	Total sample size	Mean no. of alleles per locus	Polymorphic loci (of 8 possible)	Mean heterozygosity ($\bar{H}$)	
				Observed heterozygosity	Expected heterozygosity
Elizabeth River					
AW	110	2.6	8	0.171 (0.056)	0.189 (0.056)
SC	88	2.5	7	0.187 (0.061)	0.192 (0.058)
GC	96	2.9	8	0.191 (0.070)	0.203 (0.065)
JC	107	2.9	7	0.213 (0.057)	0.231 (0.055)
PC	79	2.8	8	0.213 (0.054)	0.219 (0.053)
ER Pooled	480	3.0	8	0.188 (0.053)	0.206 (0.053)
York River					
CI	112	2.8	7	0.214 (0.071)	0.207 (0.065)

low expected genotypic values (< 5) and applying a sequential Bonferroni correction (Rice 1989), tests for Hardy-Weinberg equilibrium for the combined ER sites and the CI site revealed only the *Fum*-A locus had significant deviations from Hardy-Weinberg expectations for the combined ER sites ($p < 0.008$)(Table 7).

After applying a sequential Bonferroni correction for multiple tests (Rice 1989), the table-wide significance value at the 95% confidence level was P = 0.006 (Table 8). Heterogeneity chi-square analyses between CI and the combined ER collection sites revealed only one significantly heterogeneous locus for this comparison (*Gpi*-A). Wright's F_{st} value for the CI-ER comparison was 0.0053 (Table 9), which was not significantly different from 0 as derived from a Markov Chain using 10,000 iterations. An UPGMA phenogram of the genetic distance between collections from the two rivers is shown in Figure 3.

Comparison Among Elizabeth River Sites

Mean observed heterozygosity among samples from the Elizabeth River collection sites ranged between $\overline{H}$= 0.171 - 0.213 and there were no significant differences in heterozygosity among sites (Table 6). SC had the lowest mean number of alleles per locus at 2.5, but its mean

heterozygosity was close to the value for the ER collections (0.187 and 0.188 respectively). Also, there were no significant differences between the observed and expected mean heterozygosities for any of the ER sites.

When considering all loci individually, and after excluding thirty tests with low expected genotypic values (< 5) and then applying the calculated table-wide sequential Bonferroni correction of P = 0.008 to all samples, the distribution of genotypes was not significantly different from Hardy-Weinberg expectations for any locus (Table 7).

Contingency chi-square analysis of heterogeneity revealed significant heterogeneity for three of the five allozyme loci (*Ldh*-B, *Gpi*-A and *Idh*-B) among the ER collection sites after reducing the table-wide 95% confidence level to P = 0.006 due to multiple testing (Rice 1989) and excluding four cells with expected values less than 5 (Table 8). The mean F_{st} value of 0.0242 was significantly different than 0 ($P \leq 0.05$, derived from a Markov Chain using 10,000 iterations)(Table 9), indicating the presence of population subdivision among these collection sites. Genetic similarity values (Rogers 1972) estimated from all allozyme loci for all pairwise comparisons between collection sites were ≥ 0.927. Nei's

Table 7a. Chi-square test for deviation of genotypic distributions from expectations of Hardy-Weinberg equilibrium for eight polymorphic loci for the combined ER sites and Catlett Islands in the York River. (* Test not applicable due to cells with low expected values, or not polymorphic; df = degrees of freedom).

	Elizabeth River sites (combined)			York River (CI)		
	X^2	df	P	X^2	df	P
Ldh-B	4.1	3	>0.05	0.8	3	>0.05
Gpi-A	4.0	1	0.04	0.2	1	>0.05
Idh-B	1.6	1	>0.05	0.4	1	>0.05
Mdh-A	*			*		
Pgm-A	0.2	1	>0.05	3.6	6	>0.05
Fum-A	89.1	1	<0.05	*		
Mpi-A	*			*		
Aat-1	*			*		

Table 7b. Chi-square test for deviation of genotypic distributions from expectations of Hardy-Weinberg equilibrium for eight polymorphic loci for five locations in the Elizabeth River. (* Test not applicable due to cells with low expected values, or not polymorphic; df = degrees of freedom).

	AW			SC			JC			GC			PC		
	X^2	df	P	X^2	df	P	X^2	df	P	X^2	df	P	X^2	df	P
Ldh-B	*			5.8	3	>0.05	*			1.5	3	>0.05	*		
Gpi-A	*			*			0.1	1	>0.05	*			*		
Idh-B	0.6	3	>0.05	1.3	1	>0.05	2.3	3	>0.05	0.4	3	>0.05	0.1	1	>0.05
Mdh-A	*			*			*			*			*		
Pgm-A	*			*			4.8	6	>0.05	5.2	3	>0.05	*		
Fum-A	*			*			*			*			*		
Mpi-A	*			*			*			*			*		
Aat-1	*			*			*			*			*		

Table 8. Contingency chi-square analysis of heterogeneity of eight polymorphic loci between collection sites located on the Elizabeth and York Rivers, and among collection sites within the Elizabeth River. (* Test not applicable due to cells with low expected values; df = degrees of freedom).

	Elizabeth River sites (combined) vs. York River(CI)				Among Elizabeth River sites (AW, SC, GC, JC, PC)			
	n	X^2	df	P	n	X^2	df	P
Ldh-B	582	1.4	2	0.1	470	25.3	4	0.001
Gpi-A	587	22.9	2	0.001	475	37.1	4	0.001
Idh-B	582	4.7	2	0.1	470	20.1	4	0.001
Mdh-A	590	2.6	2	0.1	478	*		
Pgm-A	585	3.8	2	0.1	473	12.3	4	0.01
Fum-A	587	4.3	2	0.1	475	*		
Mpi-A	590	5.6	3	0.05	478	5.5	4	0.05
Aat-1	590	8.6	2	0.01	478	*		

Table 9. Wright's F statistics based on allozyme allele frequencies for three hierarchical levels of population differentiation: 1. Combined ER sites vs. CI; 2. Sites within the ER and 3. Individual ER sites vs. CI.

	F_{st}	F_{is}	F_{it}
Combined ER vs. CI	0.0053	0.0612	0.0662
Among ER sites	0.0242	0.0554	0.0783
SC-CI	0.0108	-0.0135	-0.0026
AW-CI	0.0188	0.0237	0.0420
GC-CI	0.0046	0.0033	0.0079
JC-CI	0.0217	0.0189	0.0402
PC-CI	0.0137	-0.0132	0.0007

(1978) genetic distances ranged among pairwise comparisons from 0.0067 (Jones Creek vs. Scuffletown Creek) to 0.0104 (Atlantic Wood vs. Jones Creek, Figure 3).

Using Wright's (1978) method to allocate hierarchical population differentiation, 0.0% of the genetic diversity was attributed to differences between the York and Elizabeth Rivers, 98.0% was attributed to within collection site variation, and 1.9% was attributed to among site variation in the Elizabeth River.

Using the private allele method of Slatkin (1985), the effective number of migrants per generation between the combined ER sites and CI, and among ER sites, was estimated to be 17.2 and 3.3, respectively.

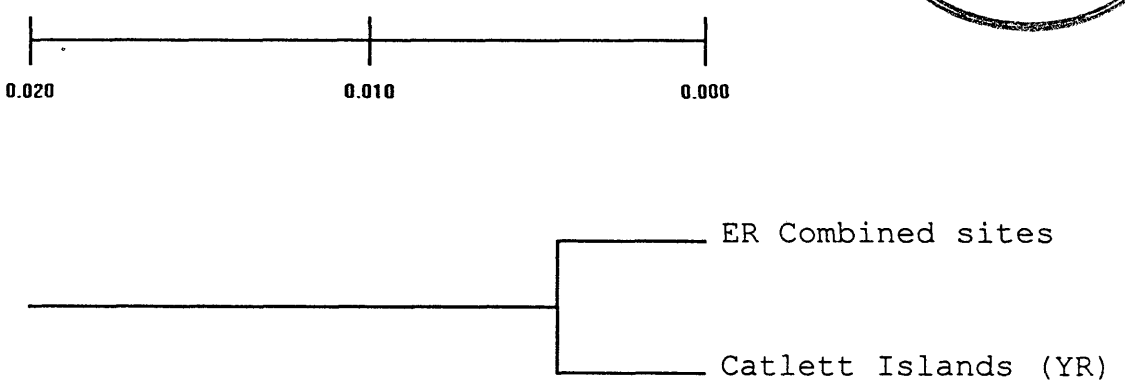
A Mantel analysis of correlation did not reject the null hypothesis that there is no association between Nei's (1978) genetic distance and geographic distance (m) (Sokal 1979), among the ER collection sites ($r = -0.6111$; two-tail proportion range 0.97 - 0.03)(Figure 4) with a calculated Z value of 116.1 and an average Z value of 126.5 after 2000 permutations of the original distance matrices.

Allozyme Data Summary

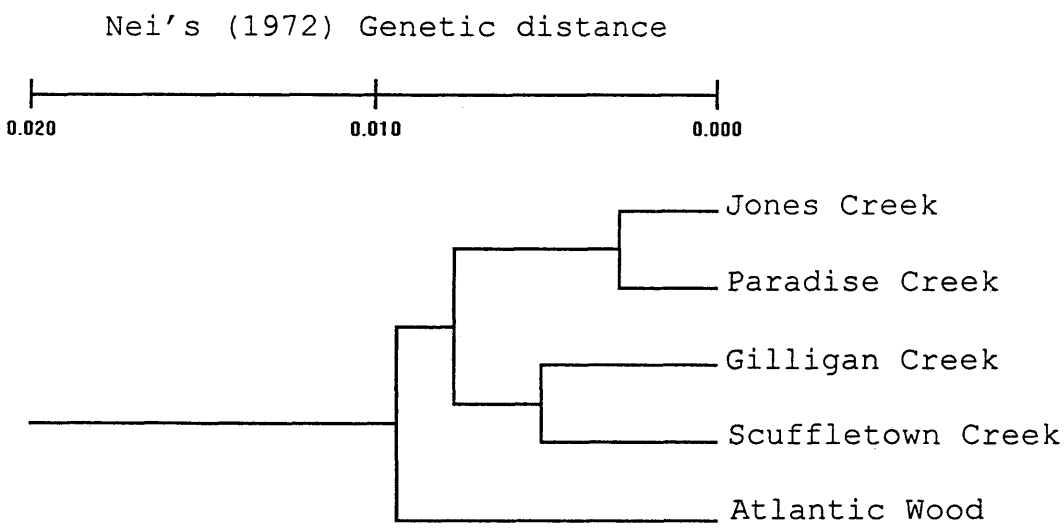
No significant differences in the levels of genetic diversity were observed between the combined ER sites and

Figure 3. Nei's (1972) genetic distance estimated from eight polymorphic allozyme loci. Upper UPGMA tree represents genetic distance between pooled Elizabeth River sites (ER) and a control site on the York River (YR). Lower UPGMA tree represents genetic distances among location sites on the Elizabeth River.

A.



B.



CI, or among the ER sites. Significant heterogeneity in allele frequency was revealed between the combined ER and CI (one locus), as well as among the Elizabeth River sites (3 loci)(Table 8). This heterogeneity resulted from significant deviations at one or two locations for each locus (Table 10b). No pattern was observed among the significant values and their occurrence between the combined ER and CI or among the Elizabeth River sites.

Mitochondrial Restriction Fragment Length Polymorphism (RFLP) Analysis

Whole Molecule Mitochondrial DNA RFLP Data

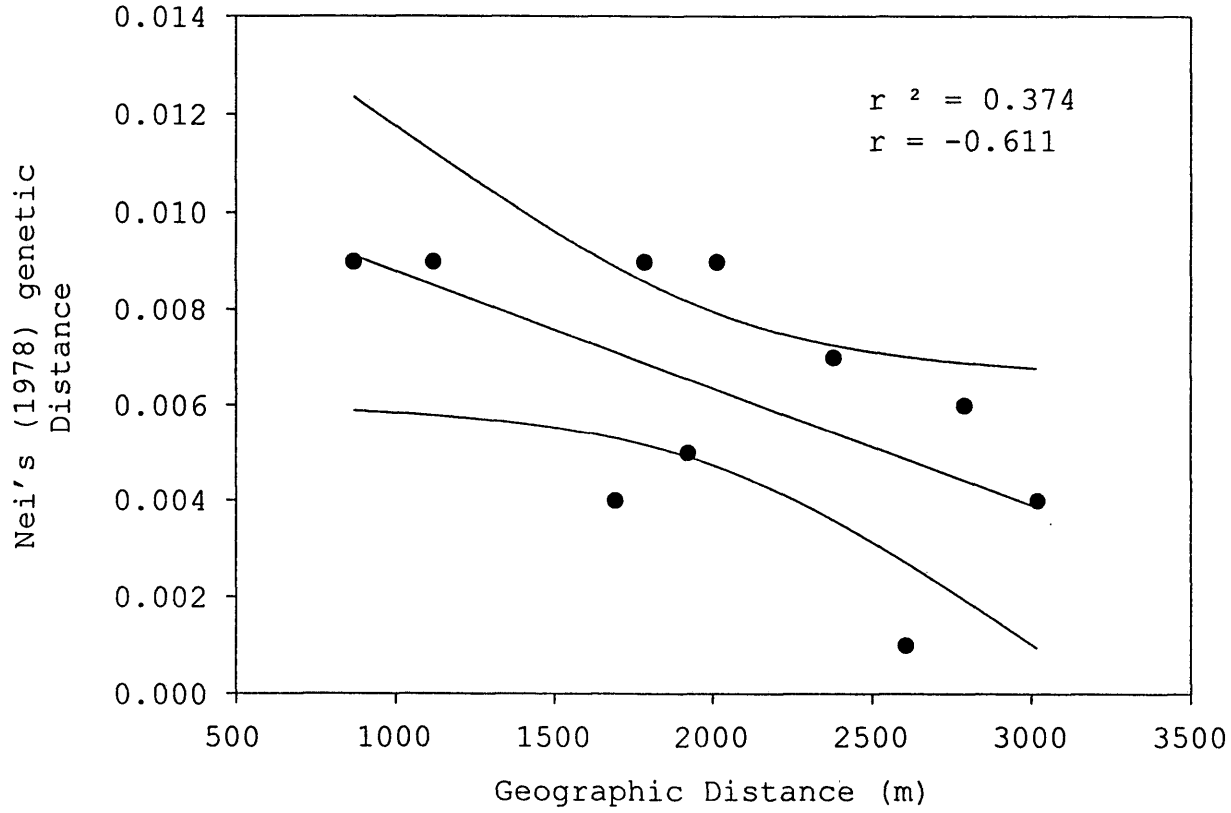
Analysis of whole molecule mtDNA from 12 *F. heteroclitus* with three restriction enzymes (*Hin*dIII, *Sma*I, and *Sst*I) revealed two composite haplotypes comprising 12 unique fragments (Figure 5). Haplotypes 1 and 2 had frequencies of 67% and 33%, respectively, among the individuals sampled. Fragment patterns observed for each of the restriction enzymes were letter-labeled according to previously reported *F. heteroclitus* mtDNA RFLP work (Brown and Chapman 1991; Gonzalez-Villaseñor and Powers 1990; Smith et al. 1998). The mean size of the *F. heteroclitus* mtDNA genome assessed from the restriction fragment profiles was approximately 16.4 Kbp.

Mitochondrial D-loop and ND-4 Restriction Fragment Data

Samples of 180 and 240 *Fundulus heteroclitus* from 1997 and 1998 collections, respectively, were analyzed for RFLPs in the D-loop and ND-4 gene regions. The size of the amplified D-loop fragment was estimated to be 1.85 Kbp while the amplified ND-4 fragment was approximately 1.95 Kbp. Five restriction enzymes (*Rsa*I, *Ecor*V, *Sma*I, *Ava*I, and *Bcl*I) revealed polymorphic restriction sites on the D-loop region and seven (*Msp*I, *Tha*I, *Hin*fI, *Ava*II, *Pal*I, *Sty*I, and *Dde*I) revealed polymorphic sites on the ND-4 region. Seventeen composite haplotypes were identified within the 1997 sample collection and twenty-five composite haplotypes were identified within the 1998 collection for a total of 31 composite haplotypes for the two years pooled. A summary of the fragment patterns and estimated sizes of each fragment are listed in Table 11. Each polymorphism could be explained by the loss or gain of one restriction site relative to the common pattern. Nucleotide sequence divergences between haplotypes ranged from 0.28% to 0.60% for the pooled samples with a mean *d* value of 0.30% (Appendix 3).

Temporal heterogeneity between samples collected at the same locations in 1997 and 1998 was evaluated via contingency chi-squared analysis and Monte-Carlo

Figure 4. Mantel correlation between Nei's (1978) genetic distance and geographic distance (m) among Elizabeth River collecting sites. The r^2 value was determined from linear regression plot (95% CI); the r value was determined from a Mantel correlation.


0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Nei's (1978) genetic Distance
r ² = 0.374
r = -0.611
500
1000
1500
2000
2500
3000
3500
Geographic Distance (m)

A. Pooled ER sites vs. Catlett Islands site

HET	*LDH*-B	*MPI*-A	*GPI*-A	*MDH*-A	*FUM*-A	*AAT*-1	*IDH*-B	*PGM*-A
ER-CI			■			■		

B. Among ER sites

HET	*LDH*-B	*MPI*-A	*GPI*-A	*MDH*-A	*FUM*-A	*AAT*-1	*IDH*-B	*PGM*-A
Within ER	■		■		■	■	■	

C. Individual ER sites vs. Catlett Islands site

HET	*LDH*-B	*MPI*-A	*GPI*-A	*MDH*-A	*FUM*-A	*AAT*-1	*IDH*-B	*PGM*-A
AW			■					
SC								
JC			■					
GC						■		
PC			■					

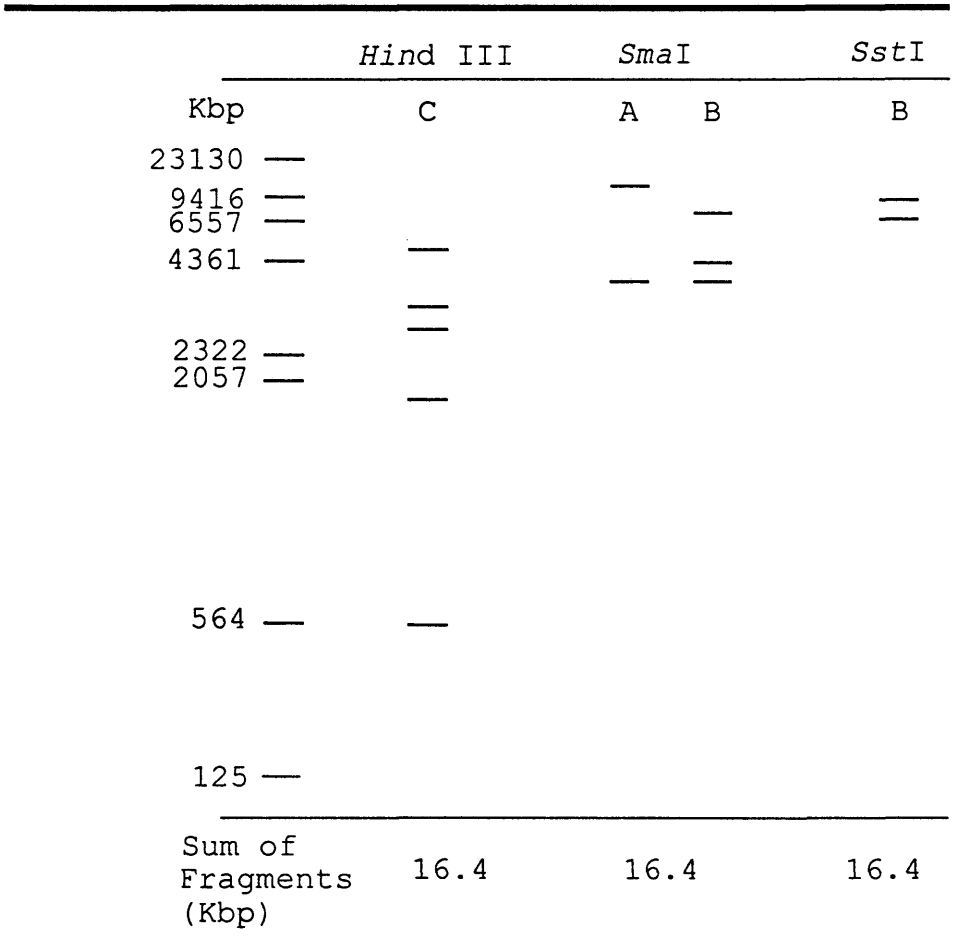
Table 10. Distribution of significant allele heterogeneity

■ Significant

□ Non-Significant

□ Test not performed after excluding cells with expected values less than five, or monomorphic loci

Figure 5. Whole mitochondrial restriction fragment profiles observed for *F. heteroclitus* from Jones Creek, Elizabeth River. Size standard (λ/*Hin*dIII) is on the first lane. Composite mtDNA haplotypes observed shown with sample size (n).



Haplotype No	n	Fragment Pattern *Hin*dIII	*Sma*I	*Sst*I
1	8	C	B	B
2	4	C	A	B
Total	12			

Table 11a. Estimated size (bp) of *Fundulus heteroclitus* mitochondrial (D-loop) restriction fragments resulting from digests with five enzymes.

	*Rsa*I				*Ecor*V		*Sma*I			*Ava*I			*Bcl*I	
	A	B	C	D	A	B	A	B	C	A	B	C	A	B
D-Loop	925			1850		1850		1850				1850	1850	
			1325		1200									1500
							1250				1300			
					650									
		800					600		700	750				
	525	525	525						550	550	550			
	400	400							475					
														325
		125							125					
Totals	1850	1850	1850	1850	1850	1850	1850	1850	1850	1850	1850	1850	1850	1850

Table 11b. Estimated size (bp) of *Fundulus heteroclitus* mitochondrial (ND-4) restriction fragments resulting from digests with seven enzymes.

	*Msp*I		*Tha*I		*Hinf*I		*Ava*II	*Pal*I			*Sty*I		*Dde*I	
	A	B	A	B	A	B	A	A	B	C	A	B	A	B
ND-4	1100	830(2)	1660	1950	500	800	925	1630	1130	1950	1660	900	925	800
	550				350	500	525		500			760	525	525
	290	290	290		300(2)	350	400	320	320		290	290	400	400
					175	300								125
					125									
Totals	1940	1950	1950	1950	1750	1950	1850	1950	1950	1950	1950	1950	1850	1850

simulations (Roff and Bentzen 1989). In all instances, no statistically significant differences were observed between the 1997 and 1998 collections at any location. This allowed for pooling of the data from both years at each locality.

Restriction enzyme analysis of the pooled samples with 12 enzymes that produced polymorphic fragment patterns revealed 31 composite mtDNA haplotypes (n = 420) among *F. heteroclitus* from the six collecting sites (Table 12). Haplotype 1 was the most common, representing 320 (76%) of all individuals sampled (Table 12). Among collection sites, the frequency of haplotype 1 varied from 61% (JC) to 84% (SC).

Haplotypes 7,17 and 2 represented 5%, 3%, and 2% of the individuals sampled, respectively (Table 12). Haplotype 7 was found in the Atlantic Wood and Jones Creek samples at a frequency of 13.5%, and was present at a frequency of 4% in Paradise Creek. This haplotype was not found at any other sites (Table 12). Fifteen composite haplotypes were unique to single individuals. Jones Creek had the highest number of unique haplotypes (40%) followed by Paradise Creek (20%), and Atlantic Wood, Gilligan Creek and Catlett Island (each with 13.3% unique haplotypes,

Table 12. Composite mtDNA haplotypes detected in *Fundulus heteroclitus* from the Elizabeth River (AW, SC, GC, JC, PC) and the York River (CI) in 1997 and 1998. Restriction enzymes (from left to right) are: *Rsa*I, *Eco*rV, *Sma*I, *Ava*I, *Bcl*I, *Msp*I, *Tha*I, *Hinf*I, *Ava*II, *Pal*I, *Sty*I, and *Dde*I.

Haplotype No.	Pattern	AW	SC	GC	JC	PC	CI	Total
1	AAAAAAAAAAAA	51	59	59	43	50	58	320
2	AAAAAAAAAABA	-	2	4	-	3	-	9
3	AAAAAAAAAABB	-	-	1	-	-	-	1
4	AAAAAAAAABAA	-	-	-	-	2	-	2
5	AAAAAAAAACAA	-	2	-	-	-	-	2
6	AAAAAAABAAAA	-	-	-	3	2	-	5
7	AAAAAABAAAAA	11	-	-	9	3	-	23
8	AAAAAABAAABA	-	-	-	1	-	-	1
9	AAAAABAAAAAA	-	2	-	-	-	-	2
10	AAAAABBAABAA	-	-	-	-	1	-	1
11	AAAABAAAAAAA	1	-	-	-	-	-	1
12	AAABAAAAAAAA	-	1	2	2	-	2	7
13	AAABAAABAAAA	-	-	-	1	-	-	1
14	AAABAABAAAAA	-	-	-	1	-	-	1
15	AAABABBAABAA	-	-	-	-	1	-	1
16	AAACAAAAAAAA	-	-	-	1	-	-	1
17	AABAAAAAAAAA	1	2	2	4	-	3	12
18	AABAAAAAAABA	-	-	-	-	1	-	1
19	AABAAAAAAABB	-	-	1	-	-	-	1
20	AABAAABAAAAA	-	-	-	2	-	-	2
21	AABAAABAAABA	-	-	-	1	-	-	1
22	AABBAAAAAAAA	1	1	-	-	2	-	4
23	AABBAABAAAAA	1	-	-	-	1	-	2
24	AACAAAAAAAAA	-	-	-	-	-	5	5
25	ABAAAAAAAAAA	1	-	-	-	-	-	1
26	ABAAAABAAAAA	1	-	-	-	-	-	1
27	BAAAAAAAAAAA	1	-	-	-	-	1	2
28	BABAAAAAAAAA	-	-	-	-	-	1	1
29	CAAAAAAAAAAA	1	1	1	-	3	-	6
30	CABAAAAAAAAA	-	-	-	1	-	-	1
31	DAAAAAAAAAAA	-	-	-	1	1	-	2
Total		70	70	70	70	70	70	420

Table 12). The distribution of composite haplotypes from all six sites is presented in Figure 6.

Mean haplotype diversity at the six sites was 0.366 (range 0.288 - 0.606). Jones Creek had the highest diversity at 0.606 followed by Paradise Creek (0.487) and Atlantic Wood (0.449)(Table 13). The Jones Creek value was not significantly different than the other values (Grubbs' test for single outlier; $T_{calc} < T_{table}$)(Newman 1995). Scuffletown Creek and Gilligan Creek had identical haplotype diversities at 0.288, close to that of the Catlett Islands (0.309). The overall mean nucleotide sequence diversity was 0.57% (range 0.31 - 1.0%) (Table 13). A trend similar to the mean haplotype diversity was evident in the mean nucleotide sequence diversities, with the value for Jones Creek (1.07%) being more than three times greater than Scuffletown Creek, Gilligan Creek, and Catlett Islands (Table 13). Here too, the Jones Creek value was not significantly different (Grubbs' test for single outlier $T_{calc} < T_{table}$)(Newman 1995).

Comparison Between the York River Site and the Combined Elizabeth River Sites

The net mean nucleotide sequence divergence between river samples was 0.015% (Table 13). Chi-square

heterogeneity analysis between the two locations using the Roff and Bentzen (1989) procedure produced chi-square values greater than the observed value 33 out of 1000 times ($0.05 > P > 0.01$)(Table 15) indicating significant spatial partitioning of composite haplotypes between CI and pooled ER collection sites.

Comparison Among Elizabeth River Sites

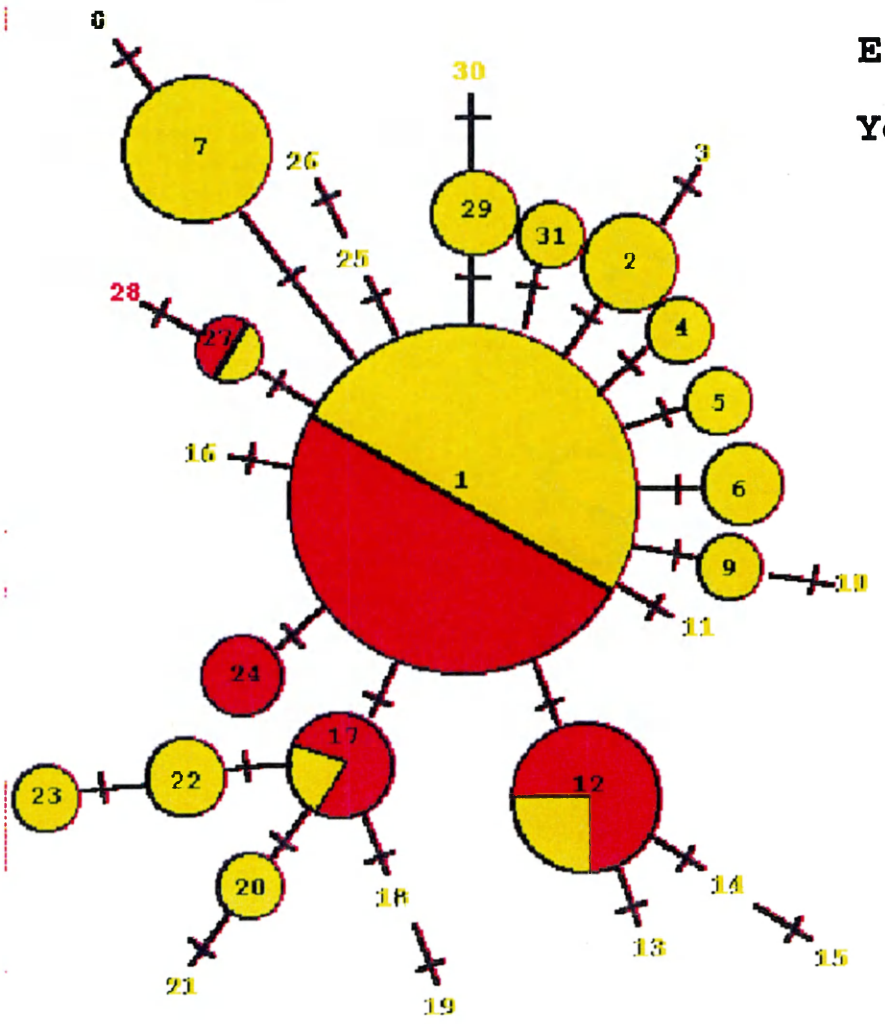
Among the ER sites, the mean net nucleotide sequence divergence was minimum (0.012%). However, this value is essentially the same as that between the combined ER samples value (0.015%)(Table 13). Chi-square tests of homogeneity between Elizabeth River samples using a Monte Carlo simulation revealed significant heterogeneity for five of ten pairwise comparisons (AW-SC; AW-GC; SC-JC; GC-JC and, JC-PC)(Table 15). All other tests were non-significant after corrections for multiple testing (Roff and Bentzen 1989).

Chi-square tests of homogeneity using Monte Carlo simulations (Roff and Bentzen 1989) between each of the Elizabeth River samples and CI, revealed significant heterogeneity for three pair-wise comparisons: AW-CI; JC-CI and, PC-CI (δ_{net}= 0.00027; 0.00037 and 0.00011, respectively).

Figure 6. Relationships among *Fundulus heteroclitus* mtDNA haplotypes (D-loop and ND-4). Areas of circles are proportional to the total number of individuals possessing the haplotype. Each crossbar represents one restriction site change. Haplotypes possessed by only one individual are listed by haplotype designation only.

a) Elizabeth and York rivers: haplotype frequencies from York River sample shown normalized to sample size

b) Within ER sites



Elizabeth River sites

York River site

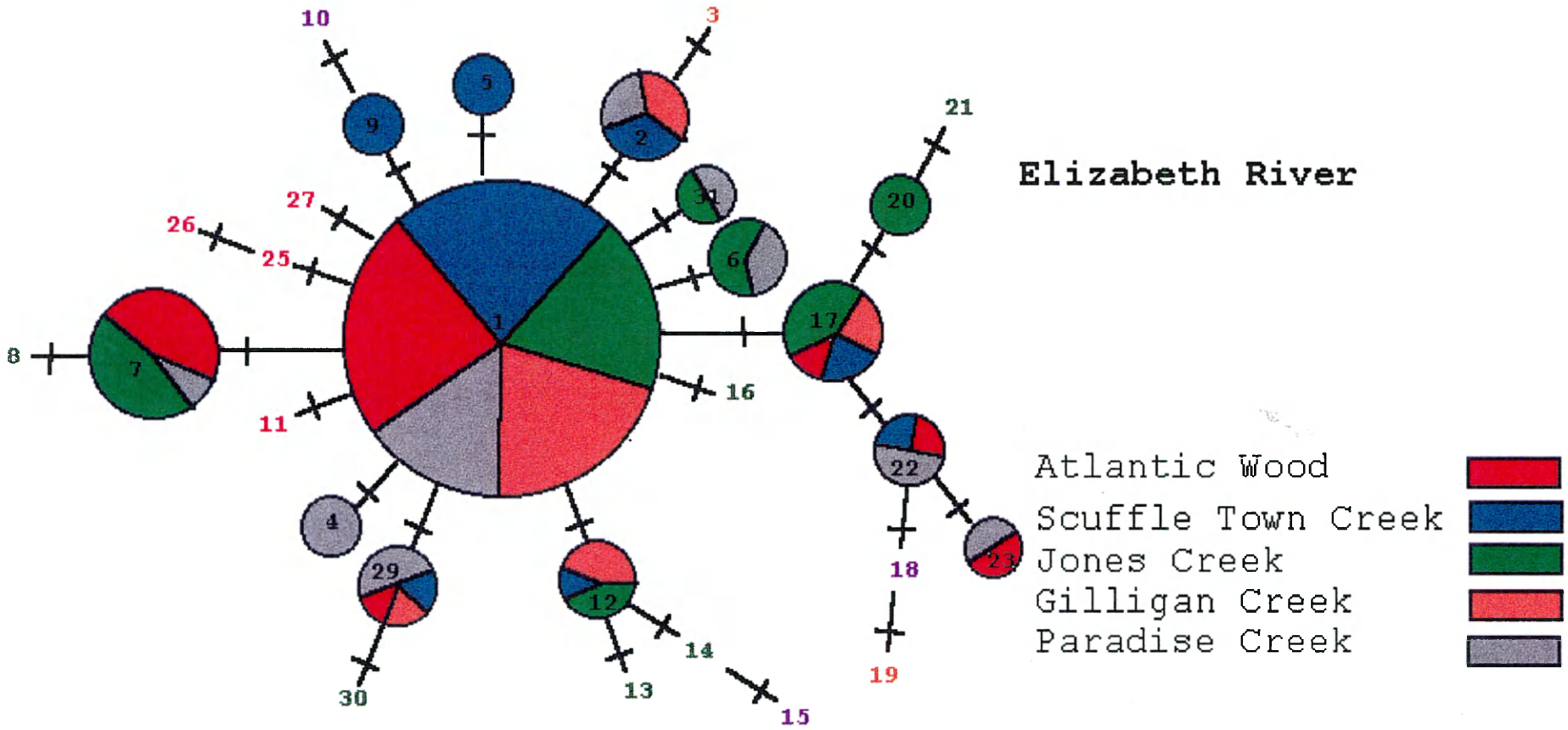

10
3
5
9
2
21
27
20
26
25
1
17
8
7
16
11
22
4
18
29
12
23
14
30
13
19
15
Elizabeth River
Atlantic Wood
Scuffle Town Creek
Jones Creek
Gilligan Creek
Paradise Creek

Table 13. Summary statistics of *Fundulus heteroclitus* mtDNA variability (D-loop plus ND-4 regions).

Sample type	Location /year	*n*	Haplotype diversity (*h*)	Nucleotide diversity (Π)	Mean Nucleotide Sequence divergence (δ)
Temporal	1997	180	0.350	0.71%	
	1998	240	0.433	0.67%	
	Pooled	420	0.402	0.59%	
Collection site	Elizabeth River				
	AW	70	0.449	0.47%	
	SC	70	0.289	0.30%	
	GC	70	0.288	0.30%	
	JC	70	0.606	1.07%	
	PC	70	0.487	0.91%	
	Pooled ER	350	0.424		
	York River				
	CI	70	0.309	0.35%	
Pairwise comparison	ER combined vs. CI	420	0.364	0.48%	0.015%
	Among ER	350	0.421	0.61%	0.012%

Table 14. Net nucleotide sequence divergence among *Fundulus heteroclitus* samples based on mtDNA RFLPs.

	AW	SC	GC	JC	PC	CI
AW	--					
SC	0.00025	--				
GC	0.00029	0.00001	--			
JC	0.00001	0.00034	0.00039	--		
PC	0.00008	0.00003	0.00006	0.00003	--	
CI	0.00027	0.00005	0.00011	0.00037	0.00011	--

Table 15. Evaluation of chi-square homogeneity of mtDNA haplotypes using Monte Carlo simulations. P values are the portion of randomized chi-square values exceeding the observed chi-square value (Roff and Bentzen, 1989).

	AW	SC	GC	JC	PC	CI
AW	-					0.0000 (0.0000)
SC	0.0010 (±0.0010)					0.0420 (±0.0063)
GC	0.0000 (0.0000)	0.6650 (±0.0149)				0.0340 (±0.0057)
JC	0.0580 (±0.0074)	0.0000 (0.0000)	0.0000 (0.0000)			0.0000 (0.0000)
PC	0.0440 (±0.0065)	0.0380 (±0.0060)	0.0200 (±0.0044)	0.0050 (±0.0022)	-	0.0000 (0.0000)
Combined ER	→	→	→	→		0.0330 (±0.0056)

A hierarchical analysis of variance (AMOVA) was completed between the Elizabeth and York rivers, among collection sites within rivers, and within collection sites. This analysis revealed no significant differences in genetic variation attributable to differences between-rivers, 3.0% due to variation among collecting sites and, 97.4% of the variance due to differences among individuals within-collection sites.

Mitochondrial Data Summary

No significant differences in levels of haplotype diversity were observed between the combined ER sites and CI, or among the ER sites. Significant heterogeneity was revealed between individual sites on the ER and CI and among the Elizabeth River sites (Table 15). No pattern was observed among the significant values and their occurrence at each of the Elizabeth River sites.

The net mean nucleotide sequence divergence estimates were similar for all pair-wise comparisons among ER sites and between the combined ER site samples and CI (Table 14).

DISCUSSION

Population genetic studies analyze the distribution of genetic variation over time and space. To be effective, the molecular markers investigated must reveal substantial variation. *Fundulus heteroclitus* has previously been reported to be among the most genetically diverse fish species (Powers et al. 1991). Mitton and Koehn (1975) found that more than 50% of 25 allozyme loci scored within a single population were polymorphic, with an average estimated heterozygosity of 0.18 per locus per individual. *Fundulus heteroclitus* nucleotide sequence divergence was estimated at 0.019, which is within the average nucleotide sequence divergence for freshwater fish (0.004 - 0.087) and marine species (0.004 - 0.029) (Gonzalez-Villaseñor and Powers 1990). Results of the present study are consistent with previous investigations in the Chesapeake Bay region, revealing high genetic diversity (Ropson et al. 1990; Smith et al. 1998; Smith et al. 1992).

The distribution of genetic variation among populations represents the combined effects of gene flow, genetic drift and selection. In the absence of gene flow, genetic drift will tend to increase genetic divergence between samples. If the migration range of *F. heteroclitus*

is as restricted as reported (Fritz et al. 1975; Lotrich 1975), differentiation between samples would be expected to occur by genetic drift and possibly from adaptation to local habitats.

Anthropogenic factors also can provide selective forces that result in genetic divergence between populations, and affect overall levels of variation within populations (Guttman 1994; Murdoch and Hebert 1994). Correlations between genetic diversity and contamination from xenobiotics of anthropogenic origin often reveal a decrease in the overall genetic diversity of the affected population relative to populations from non-contaminated habitats (Mulvey and Diamond 1991). In this study, mean heterozygosity and polymorphism values for the allozyme loci investigated in six *F. heteroclitus* samples were homogeneous. Similarly, there were no significant differences in haplotype diversity of mtDNA gene regions among the six samples.

Murdoch and Hebert (1994) observed significant differences in the haplotypic diversity of mtDNA surveyed on the brown bullhead (*Ameiurus nebulosus*) from nine lower Great Lakes sites, that represented a range in sediment contamination. In that study, genetic diversity was always lower in populations inhabiting PAH-contaminated sites and

the authors concluded that the most parsimonious explanation for the reduced diversity was the occurrence of bottlenecks caused by sudden environmental changes. In the present study, no significant difference was found in the levels of genetic variation (allozyme heterozygosity and mtDNA haplotypic diversity) among sites with different levels of PAH contamination.

Temporal Heterogeneity

Analysis of temporal genetic variation in allozyme and mtDNA haplotype frequencies can be used to identify the presence of stochastic effects such as small effective population sizes, high variances in reproductive success or non-random sampling within collection sites (Gold et al. 1993). Contingency chi-square tests and Monte Carlo simulations of the allozyme and mtDNA allele frequencies, respectively, showed no heterogeneity between samples collected in 1997 and 1998 at any of the six locations for any locus. Thus, population genetic characters of *F. heteroclitus* appear to be stable over small time scales.

Temporal stability of allelic frequencies has been reported for *F. heteroclitus* populations on annual or biannual collections over a six year sampling period (1969 - 1975) at various locations along the east coast (Powers

and Place 1978). Temporally replicated samples increase the ability to discern artifacts that may confound the genetic signals, especially if the differences between samples are modest (Waples 1998). An added benefit of stability of allele frequencies over time is the ability to pool sample information collected over multiple years thus increasing the power of statistical tests by the increase in sample size. Unlike Powers and Place (1978), the extent of data pooling for this study was limited to two years. Since *F. heteroclitus* has a lifespan of approximately three years, and are capable of approximately two reproductive cycles per annum (Kneib 1986), two years was not a sufficient time to guarantee complete sample replacement at each location.

Geographic Population Structure of *Fundulus heteroclitus*

Results of this genetic analysis of *F. heteroclitus* inhabiting the southern branch of the Elizabeth River agree with previous studies of the species in this region using similar molecular markers. RFLP analysis of whole mtDNA using three restriction enzymes (*Hin*dIII, *Sma*I and *Sst*I), demonstrated that mtDNA restriction profiles of *F. heteroclitus* samples from the southern branch of the Elizabeth River, VA are of the 'southern' form as reported

by Gonzalez-Villaseñor and Powers (1990). These restriction profiles were also reported for Shady Side, MD (Brown and Chapman 1991) and Smithfield, VA (Smith et al. 1998). Allozyme frequencies for the eight polymorphic nuclear gene loci surveyed in this study were similar to those described for *F. heteroclitus* from Cape Charles, VA, (Cashon et al. 1981; Powers and Place 1978; Ropson et al. 1990), and the mouth of the James River (Smith et al. 1992).

Population Structure of *Fundulus heteroclitus* from the Elizabeth and York rivers

The high degree of genetic diversity in *Fundulus heteroclitus* provided many characters to evaluate the spatial partitioning of genetic variation. Significant genetic heterogeneity was observed between samples from the Elizabeth and York rivers at two allozyme loci, *Gpi*-A and *Aat*-1. Significant heterogeneity in the distribution of composite mtDNA haplotypes was also observed between samples from the Elizabeth and York rivers.

Significant genetic heterogeneity was observed among samples from the Elizabeth River. Four allozyme loci (*Ldh*-B, *Gpi*-A, *Idh*-B and, *Pgm*-A) exhibited significant heterogeneity. However, no consistent pattern of variation

was noted in the samples relative to PAH concentrations or geographic distance. In each case, a different sample was responsible for the observed heterogeneity. Significant heterogeneity in the distribution of composite mtDNA haplotypes was also observed (ER samples).

When all six samples were surveyed for genetic heterogeneity, significant differences in the distribution of allozyme and composite haplotype frequencies occurred at about the same frequency between all samples regardless of location. Significant heterogeneity values were attributed to deviant allele frequencies for one locus at one collection location, and there was no consistent pattern (by locus or location) to these deviations.

The results from this study generally agree with other genetic analyses of *F. heteroclitus* on a microgeographic scale. Brown and Chapman (1991) found that levels of mtDNA variation were greater within samples than between samples located along an 8.4 km Maryland salt marsh. In this study, Brown and Chapman (1991) surveyed a total of 480 *F. heteroclitus* with only five restriction enzymes. A chi-square simulation (Roff and Bentzen 1989) of their published data revealed chi-square values greater than the observed values 59 out of 1000 times, implying near significant heterogeneity among the eight samples. On a

much broader scale, Gonzalez-Villaseñor and Powers (1990) provided evidence of homogeneity within each of the two reported subspecies of *F. heteroclitus*.

In the present study, no correlations were observed between genetic divergence for the nuclear (allozyme) or mitochondrial markers (ND4/D-loop) and geographical distance. This implies that the gene flow among these samples is sufficient to overcome local genetic differentiation resulting from genetic drift and natural selection (Brown and Chapman 1991). Estimates of the number of migrants among samples based on allozyme F_{st} values (0.0242 $P < 0.05$) were all ≥ 3.3, such relatively high rates of migration will tend to prevent genetic divergence from developing among populations (Wright 1978). However, it is theoretically possible to maintain allele frequency differences even in the face of substantial migration between populations (Allendorf and Phelps 1981).

Hedgecock (1994) suggested that even in species considered genetically homogeneous over large regions, microspatial heterogeneity is often revealed through examination on a small geographic scale. Such differences may be attributed to inbreeding, local environmental variables, stochastic variability in reproductive success, and temporary barriers to gene flow (Hedgecock 1994). The

lack of significant differences of mean heterozygosities among samples and the lack of significant deviations from Hardy-Weinberg equilibrium suggests that the observed heterogeneity within the combined ER samples is not the result of inbreeding.

In some instances, shifts in the allele frequencies of local populations have been attributed to the presence of pollutants in the environment (Guttman 1994; Mulvey and Diamond 1991). High concentrations of creosote derived PAHs have been reported for the AW site, and Williams (1994) provided cardiovascular evidence to suggest that *F. heteroclitus* from AW are resistant to creosote-amended sediment. Furthermore, breeding studies indicated that this resistance might have a genetic basis. In the present study, allele frequencies for the eight nuclear loci and mtDNA haplotype frequencies did not reveal significant differentiation between *F. heteroclitus* from AW and other collection localities.

Williams' (1994) suggestion of a genetically based PAH resistance in AW mummichog and the lack of genetic divergence of these samples appear to be contradictory. However, the two data sets may be compatible. The molecular genetic markers observed in this study may not be linked to the factors providing resistance. In addition,

the findings from this study do not rule out the potential for maternally-based resistance, as suggested by Williams (1994).

CONCLUSIONS

Polymorphic molecular markers were used to survey the genetic diversity and divergence of five samples of *Fundulus heteroclitus* collected in locations along the southern branch of the Elizabeth River VA (ER), a heavily industrialized area, and one from the Catlett islands (CI) a pristine collection site on the York River VA. Offspring crosses (F_1) of *F. heteroclitus* from CI and Atlantic Wood on the ER, exhibited differences in the levels of resistance to sediments containing high concentrations of creosote. Because the F_1 individuals had not been exposed to creosote, the differential resistance to creosote of the various crosses was attributed to have a genetic basis.

Genetic surveys of the nuclear and mitochondrial markers revealed significant genetic variability among locations within the ER and between the York River and combined ER locations. However, there were no patterns in the distribution of genetic variation among particular loci or composite haplotypes to sample locality. A contingency chi-square analysis of both molecular markers revealed temporal stability over the two-year study.

Population heterogeneity among *F. heteroclitus* samples varied according to the type of structure analyzed.

Samples within the Elizabeth River showed significant heterogeneity at microspatial scales. Heterogeneity may be the result of differences in reproductive success among individuals at a site or the cumulative effects of local environmental and spatial variables affecting these non-migratory species. There was no relationship between genetic distance, and geographic distance or PAH contamination.

The results of the present study indicate that further research is needed to fully understand the genetic mechanisms involved in the observed resistance to creosote in *F. heteroclitus* inhabiting Atlantic Wood. From a biological perspective, patterns of over-wintering migration and summer relocation of *F. heteroclitus* need to be understood so that analyses of genetic population structure can be used in perspective. From a genetic perspective, molecular markers targeting non-coding, hypervariably loci, should be used as an alternative to enzyme-coding loci, which may be under the influence of selection but may not be affected by creosote contamination.

Future Research

It has been reported that *F. heteroclitus* does not disperse much throughout their life; however, results from this study suggest that migration does occur among locations on the Elizabeth River and on larger scales (YR/ER). Knowledge of the movements of *F. heteroclitus* for a single generation is critical to understand their population structure. A tag and release study of *F. heteroclitus* from Atlantic Wood and from several, less contaminated areas of the Elizabeth River should be conducted over a two year period to test the dispersal ability and site fidelity of these fish.

To further assess whether the observed resistance to creosote has a genetic basis, additional breeding experiments should be performed. These experiments should be extended to F_2 and F_3 generations to determine if resistance levels change if challenge to toxicant exposure is removed in multiple generations.

Population genetic analyses should be extended to several more nuclear loci to increase the potential to find molecular markers that may be linked to genes responsible for resistance.

APPENDICES

Appendix 1a.

D-loop		Region size: 1.95Kbp Primer: 25 bp
Enzyme	**cut**	**Fragment size(s) {bp}**
Alu I	Y	880;246;290;80
Ava I	Y	1080; 700; 380
Ava II	Y	1450; 390
*Bam*H I	N	~ 1900
Ban I	Y	~ 1330; ~ 490
Bcl I	N	~ 1900
Bgl II	N	~ 1900
Bgl I	N?	~ 1900
Bsp 106 I	N	~ 1900
BssH II	N	~ 1900
BstE II	N	~ 1900
BstN I	Y	
Bsu 36 I	N	~ 1900
Cla I	N	~ 1900
Dde I	Y	
EcoRV	Y	690; 1100 partial digestions: (259; 400)
Hae III	Y	920;610;250(poor estimate) — 980; 515; 230 (good estimate)
Hinc II	Y	984; 738
Hind III	N	~ 1900
Hinf I	Y	1020; 396;344
Mbo I	N	~ 1900
Msp I	Y	720;260;240; small fragments
Nde I	N	
Pal I	Y	861;615;246
Pst I	N	

Enzyme	**cut**	**Fragment size(s) {bp}**
Rsa I	Y	861;492;440 (poor estimate) – 940; 500; 400 (good estimate).
Sma I	Y	1353;500 or – 1280; 492
Sst I	N	~ 1720
Sst II	Y	1580; 200 (in small fragments)
Xba I	N	~ 1720
Xho I	Y	1200;600 – 1150; 600

Appendix 1b.

ND-4		Molecule size: ~ 1.85 Kbp Primer: 20 bp
Enzyme	**cut**	**Fragment size(s) {bp}**
Alu I	Y	~ 460; 90,95, 201, 224
Apa I	N	
Ava I	Y	
Ava II	Y	~ 800; ~ 900
BamH I	N	
Bcl I	N	
Bcl I	N	
Bgl I	N	
Bsm I	N	
Bste II	N	
BstN I	Y	
Cla I	Y	
Dra I	N	
Dde I	Y	
EcoR I	N	
EcoR V	Y	
Hae III	Y	
Hha I	Y	
Hinc II	Y	
Hinf I	Y	
Hpa I	N	
Kpn I	N	
Mbo I	Y	
Mse I	Y	
Msp I	Y	490; 250
Not I	Y?	

Nsi I	N	
Enzyme	**cut**	**Fragment size(s) {bp}**
Pal I	Y	
Rsa I	Y	~ 900; ~ 600; ~ 400
Sma I	N	
Sst I	N	
Stu I	N	
Sty I	Y	
Taq I	N	
Tha I	Y	

Appendix 2. Allozyme summary for 1997 and 1998 data

1997-GILLIGAN CREEK

	Locus and sample size							
Allele	LDHB 34	GPIA 34	IDHB 34	MDHA 34	PGMA 34	FUMA 34	MPIA 34	AAT1 34
1	.000	.000	.044	.015	.000	.015	.000	.000
2	.750	.088	.750	.971	.838	.985	.971	1.000
3	.250	.912	.206	.015	.118	.000	.000	.000
4	.000	.000	.000	.000	.044	.000	.029	.000
H	.375	.161	.393	.058	.282	.029	.057	.000
H(unb)	.381	.163	.399	.058	.286	.029	.058	.000
H(D.C.)	.265	.176	.441	.059	.265	.029	.059	.000

Mean heterozygosity per locus (biased estimate) = .169 (S.E. .056)
Mean heterozygosity per locus (unbiased estimate) = .172 (S.E. .057)
Mean heterozygosity per locus (direct-count estimate)= .162(S.E. .054)

Mean number of alleles per locus = 2.25 (S.E. .25)
Percentage of loci polymorphic (no criterion) = 87.50

1997-JONES CREEK

	Locus and sample size							
Allele	LDHB 57	GPIA 57	IDHB 57	MDHA 57	PGMA 56	FUMA 57	MPIA 57	AAT1 57
1	.000	.000	.018	.000	.000	.035	.009	.026
2	.798	.298	.807	1.000	.830	.965	.886	.974
3	.202	.702	.175	.000	.143	.000	.070	.000
4	.000	.000	.000	.000	.027	.000	.035	.000
H	.322	.419	.318	.000	.289	.068	.209	.051
H(unb)	.325	.422	.320	.000	.292	.068	.211	.052
H(D.C.)	.333	.351	.316	.000	.232	.035	.123	.053

Mean heterozygosity per locus (biased estimate) = .209 (S.E. .054)
Mean heterozygosity per locus (unbiased estimate) = .211 (S.E. .055)
Mean heterozygosity per locus (direct-count estimate)=.180 (S.E. .051)

Mean number of alleles per locus = 2.38 (S.E. .32)
Percentage of loci polymorphic (no criterion) = 87.50

1997-ATLANTIC WOOD

	Locus and sample size							
Allele	LDHB 57	GPIA 58	IDHB 58	MDHA 58	PGMA 58	FUMA 58	MPIA 58	AAT1 58
1	.000	.000	.000	.017	.000	.017	.000	.026
2	.921	.233	.603	.983	.905	.983	.940	.966
3	.079	.767	.397	.000	.026	.000	.000	.009
4	.000	.000	.000	.000	.069	.000	.060	.000
H	.145	.357	.479	.034	.175	.034	.113	.067
H(unb)	.147	.360	.483	.034	.177	.034	.114	.068
H(D.C.)	.123	.431	.414	.034	.155	.034	.121	.034

Mean heterozygosity per locus (biased estimate) = .176 (S.E. .057)
Mean heterozygosity per locus (unbiased estimate) = .177 (S.E. .057)
Mean heterozygosity per locus (direct-count estimate)=.168 (S.E. .058)

Mean number of alleles per locus = 2.25 (S.E. .16)
Percentage of loci polymorphic (no criterion) =100.00

1997-SCUFFLETOWN CREEK

	Locus and sample size							
Allele	LDHB 36	GPIA 36	IDHB 36	MDHA 36	PGMA 36	FUMA 36	MPIA 36	AAT1 36
1	.069	.000	.056	.000	.014	.069	.000	.000
2	.750	.139	.750	1.000	.889	.931	.958	.986
3	.181	.861	.194	.000	.056	.000	.028	.014
4	.000	.000	.000	.000	.042	.000	.014	.000
H	.400	.239	.397	.000	.205	.129	.081	.027
H(unb)	.406	.243	.402	.000	.208	.131	.082	.028
H(D.C.)	.444	.278	.333	.000	.222	.083	.083	.028

Mean heterozygosity per locus (biased estimate) = .185 (S.E. .055)
Mean heterozygosity per locus (unbiased estimate) = .187 (S.E. .055)
Mean heterozygosity per locus (direct-count estimate)=.184 (S.E. .057)

Mean number of alleles per locus = 2.50 (S.E. .33)
Percentage of loci polymorphic (no criterion) = 87.50

1997-PARADISE CREEK

	Locus and sample size							
Allele	LDHB 31	GPIA 31	IDHB 30	MDHA 31	PGMA 31	FUMA 31	MPIA 31	AAT1 31
1	.000	.000	.000	.000	.000	.016	.016	.145
2	.790	.258	.750	1.000	.839	.984	.887	.855
3	.210	.742	.250	.000	.065	.000	.000	.000
4	.000	.000	.000	.000	.097	.000	.097	.000
H	.331	.383	.375	.000	.283	.032	.203	.248
H(unb)	.337	.389	.381	.000	.288	.032	.207	.252
H(D.C.)	.419	.452	.433	.000	.194	.032	.226	.161

Mean heterozygosity per locus (biased estimate) = .232 (S.E. .052)
Mean heterozygosity per locus (unbiased estimate) = .236 (S.E. .053)
Mean heterozygosity per locus (direct-count estimate)=.240 (S.E. 063)

Mean number of alleles per locus = 2.13 (S.E. .23)
Percentage of loci polymorphic (no criterion) = 87.50

1997-CATLETT ISLAND

	Locus and sample size							
Allele	LDHB 62	GPIA 62	IDHB 62	MDHA 62	PGMA 62	FUMA 62	MPIA 62	AAT1 62
1	.008	.000	.056	.000	.000	.048	.000	.097
2	.798	.040	.669	1.000	.823	.944	.968	.903
3	.194	.960	.274	.000	.024	.008	.016	.000
4	.000	.000	.000	.000	.153	.000	.016	.000
H	.325	.077	.474	.000	.299	.107	.063	.175
H(unb)	.328	.078	.477	.000	.302	.108	.063	.176
H(D.C.)	.339	.081	.484	.000	.323	.113	.065	.161

Mean heterozygosity per locus (biased estimate) = .190 (S.E. .057)
Mean heterozygosity per locus (unbiased estimate) = .192 (S.E. .058)
Mean heterozygosity per locus (direct-count estimate)=.196 (S.E. .059)

Mean number of alleles per locus = 2.50 (S.E. .27)
Percentage of loci polymorphic (no criterion) = 87.50

1998-GILLIGAN CREEK

	Locus and sample size							
Allele	LDHB 62	GPIA 62	IDHB 62	MDHA 62	PGMA 62	FUMA 62	MPIA 62	AAT1 62
1	.008	.000	.040	.040	.000	.000	.016	.024
2	.710	.040	.613	.960	.742	1.000	.911	.960
3	.282	.960	.347	.000	.121	.000	.024	.016
4	.000	.000	.000	.000	.137	.000	.048	.000
H	.417	.077	.502	.077	.416	.000	.166	.078
H(unb)	.420	.078	.507	.078	.419	.000	.168	.079
H(D.C.)	.548	.048	.516	.016	.371	.000	.081	.081

Mean heterozygosity per locus (biased estimate) = .217 (S.E. .069)
Mean heterozygosity per locus (unbiased estimate) = .219 (S.E. .070)
Mean heterozygosity per locus (direct-count estimate)=.208 (S.E. .082)

Mean number of alleles per locus = 2.63 (S.E. .32)
Percentage of loci polymorphic (no criterion) = 87.50

1998-JONES CREEK

	Locus and sample size							
Allele	LDHB 46	GPIA 48	IDHB 46	MDHA 48	PGMA 46	FUMA 48	MPIA 48	AAT1 48
1	.000	.000	.065	.010	.022	.021	.000	.083
2	.815	.208	.717	.990	.848	.979	.969	.896
3	.185	.792	.217	.000	.054	.000	.021	.021
4	.000	.000	.000	.000	.076	.000	.010	.000
H	.301	.330	.434	.021	.272	.041	.061	.190
H(unb)	.305	.333	.439	.021	.275	.041	.062	.192
H(D.C.)	.326	.292	.348	.021	.304	.000	.063	.208

Mean heterozygosity per locus (biased estimate) = .206 (S.E. .054)
Mean heterozygosity per locus (unbiased estimate) = .208 (S.E. .055)
Mean heterozygosity per locus (direct-count estimate)=.195 (S.E. .051)

Mean number of alleles per locus = 2.63 (S.E. .26)
Percentage of loci polymorphic (no criterion) =100.00

1998-ATLANTIC WOOD

	Locus and sample size							
Allele	LDHB 52	GPIA 52	IDHB 52	MDHA 52	PGMA 52	FUMA 52	MPIA 52	AAT1 52
1	.010	.000	.010	.000	.010	.048	.000	.000
2	.885	.135	.587	1.000	.798	.952	.885	.990
3	.106	.865	.404	.000	.077	.000	.000	.010
4	.000	.000	.000	.000	.115	.000	.115	.000
H	.206	.233	.493	.000	.344	.092	.204	.019
H(unb)	.208	.235	.498	.000	.347	.092	.206	.019
H(D.C.)	.115	.115	.558	.000	.327	.096	.154	.019

Mean heterozygosity per locus (biased estimate) = .199 (S.E. .059)
Mean heterozygosity per locus (unbiased estimate) = .201 (S.E. .059)
Mean heterozygosity per locus (direct-count estimate)= .173(S.E. .065)

Mean number of alleles per locus = 2.38 (S.E. .32)
Percentage of loci polymorphic (no criterion) = 87.50

1998-SCUFFLETOWN CREEK

	Locus and sample size							
Allele	LDHB 49	GPIA 49	IDHB 49	MDHA 52	PGMA 52	FUMA 49	MPIA 52	AAT1 52
1	.092	.000	.061	.000	.010	.092	.000	.000
2	.724	.102	.724	1.000	.856	.908	.952	1.000
3	.184	.898	.214	.000	.038	.000	.038	.000
4	.000	.000	.000	.000	.096	.000	.010	.000
H	.433	.183	.425	.000	.257	.167	.092	.000
H(unb)	.437	.185	.430	.000	.259	.169	.093	.000
H(D.C.)	.510	.204	.347	.000	.288	.061	.096	.000

Mean heterozygosity per locus (biased estimate) = .195 (S.E. .060)
Mean heterozygosity per locus (unbiased estimate) = .197 (S.E. .061)
Mean heterozygosity per locus (direct-count estimate)=.188 (S.E. .065)

Mean number of alleles per locus = 2.38 (S.E. .38)
Percentage of loci polymorphic (no criterion) = 75.00

1998-PARADISE CREEK

	Locus and sample size							
Allele	LDHB 46	GPIA 48	IDHB 46	MDHA 48	PGMA 46	FUMA 48	MPIA 48	AAT1 48
1	.000	.000	.065	.010	.022	.021	.000	.083
2	.815	.208	.717	.990	.848	.979	.969	.896
3	.185	.792	.217	.000	.054	.000	.021	.021
4	.000	.000	.000	.000	.076	.000	.010	.000
H	.301	.330	.434	.021	.272	.041	.061	.190
H(unb)	.305	.333	.439	.021	.275	.041	.062	.192
H(D.C.)	.326	.292	.348	.021	.304	.000	.063	.208

Mean heterozygosity per locus (biased estimate) = .206 (S.E. .054)
Mean heterozygosity per locus (unbiased estimate) = .208 (S.E. .055)
Mean heterozygosity per locus (direct-count estimate)=.195 (S.E. .051)

Mean number of alleles per locus = 2.63 (S.E. .26)
Percentage of loci polymorphic (no criterion) =100.00

1998-CATLETT ISLANDS

	Locus and sample size							
Allele	LDHB 50	GPIA 50	IDHB 50	MDHA 50	PGMA 50	FUMA 50	MPIA 50	AAT1 50
1	.040	.000	.070	.000	.010	.020	.000	.060
2	.720	.050	.600	1.000	.730	.980	.970	.880
3	.240	.950	.330	.000	.100	.000	.020	.060
4	.000	.000	.000	.000	.160	.000	.010	.000
H	.422	.095	.526	.000	.431	.039	.059	.218
H(unb)	.427	.096	.532	.000	.436	.040	.059	.221
H(D.C.)	.420	.100	.660	.000	.460	.040	.060	.160

Mean heterozygosity per locus (biased estimate) = .224 (S.E. .073)
Mean heterozygosity per locus (unbiased estimate) = .226 (S.E. .074)
Mean heterozygosity per locus (direct-count estimate)=.237 (S.E. .086)

Mean number of alleles per locus = 2.63 (S.E. .32)
Percentage of loci polymorphic (no criterion) = 87.50

Appendix 3. Estimates of evolutionary distance from restriction site data of 31 composite haplotypes.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.0000														
2	0.0076	0.0000													
3	0.0158	0.0082	0.0000												
4	0.0070	0.0146	0.0228	0.0000											
5	0.0074	0.0156	0.0243	0.0152	0.0000										
6	0.0348	0.0448	0.0666	0.0415	0.0442	0.0000									
7	0.0074	0.0156	0.0243	0.0152	0.0166	0.0442	0.0000								
8	0.0156	0.0080	0.0168	0.0234	0.0254	0.0549	0.0081	0.0000							
9	0.0152	0.0234	0.0322	0.0232	0.0256	0.0517	0.0256	0.0344	0.0000						
10	0.0348	0.0438	0.0533	0.0232	0.0512	0.0736	0.0256	0.0344	0.0152	0.0000					
11	0.0086	0.0170	0.0259	0.0157	0.0167	0.0461	0.0167	0.0255	0.0245	0.0450	0.0000				
12	0.0076	0.0179	0.0268	0.0146	0.0156	0.0448	0.0156	0.0265	0.0234	0.0438	0.0170	0.0000			
13	0.0448	0.0580	0.0813	0.0515	0.0549	0.0097	0.0549	0.0690	0.0625	0.0852	0.0571	0.0379	0.0000		
14	0.0156	0.0265	0.0360	0.0234	0.0254	0.0549	0.0081	0.0190	0.0344	0.0344	0.0255	0.0080	0.0482	0.0000	
15	0.0438	0.0554	0.0658	0.0315	0.0610	0.0852	0.0344	0.0461	0.0234	0.0076	0.0547	0.0376	0.0801	0.0276	0.0000
16	0.0179	0.0351	0.0450	0.0249	0.0265	0.0580	0.0265	0.0445	0.0343	0.0554	0.0281	0.0085	0.0493	0.0172	0.0476
17	0.0076	0.0179	0.0268	0.0146	0.0156	0.0448	0.0156	0.0265	0.0234	0.0438	0.0170	0.0179	0.0580	0.0265	0.0554
18	0.0179	0.0085	0.0175	0.0249	0.0265	0.0580	0.0265	0.0172	0.0343	0.0554	0.0281	0.0351	0.0792	0.0445	0.0742
19	0.0268	0.0175	0.0092	0.0338	0.0360	0.0813	0.0360	0.0268	0.0438	0.0658	0.0378	0.0450	0.1047	0.0552	0.0857
20	0.0156	0.0265	0.0360	0.0234	0.0254	0.0549	0.0081	0.0190	0.0344	0.0344	0.0255	0.0265	0.0690	0.0190	0.0461
21	0.0265	0.0172	0.0268	0.0343	0.0370	0.0690	0.0190	0.0091	0.0461	0.0461	0.0374	0.0445	0.0915	0.0373	0.0650
22	0.0179	0.0351	0.0450	0.0249	0.0265	0.0580	0.0265	0.0445	0.0343	0.0554	0.0281	0.0085	0.0493	0.0172	0.0476
23	0.0265	0.0445	0.0552	0.0343	0.0370	0.0690	0.0190	0.0373	0.0461	0.0461	0.0374	0.0172	0.0605	0.0091	0.0376
24	0.0142	0.0240	0.0322	0.0203	0.0215	0.0479	0.0215	0.0318	0.0284	0.0467	0.0228	0.0240	0.0601	0.0318	0.0576
25	0.0086	0.0170	0.0259	0.0157	0.0167	0.0461	0.0167	0.0255	0.0245	0.0450	0.0312	0.0170	0.0571	0.0255	0.0547
26	0.0167	0.0255	0.0351	0.0245	0.0265	0.0564	0.0092	0.0180	0.0356	0.0356	0.0403	0.0255	0.0682	0.0180	0.0453
27	0.0070	0.0146	0.0228	0.0141	0.0152	0.0415	0.0152	0.0234	0.0232	0.0443	0.0157	0.0146	0.0515	0.0234	0.0533
28	0.0146	0.0249	0.0338	0.0219	0.0234	0.0515	0.0234	0.0343	0.0315	0.0533	0.0240	0.0249	0.0645	0.0343	0.0650
29	0.0074	0.0156	0.0243	0.0152	0.0166	0.0442	0.0166	0.0254	0.0256	0.0512	0.0167	0.0156	0.0549	0.0254	0.0610
30	0.0156	0.0265	0.0360	0.0234	0.0254	0.0549	0.0254	0.0370	0.0344	0.0610	0.0255	0.0265	0.0690	0.0370	0.0737
31	0.0166	0.0254	0.0347	0.0256	0.0291	0.0557	0.0291	0.0386	0.0400	0.0800	0.0265	0.0254	0.0673	0.0386	0.0913

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12															
13															
14															
15															
16	0.0000														
17	0.0351	0.0000													
18	0.0596	0.0085	0.0000												
19	0.0710	0.0175	0.0090	0.0000											
20	0.0445	0.0080	0.0172	0.0268	0.0000										
21	0.0703	0.0172	0.0087	0.0184	0.0091	0.0000									
22	0.0221	0.0085	0.0221	0.0320	0.0172	0.0316	0.0000								
23	0.0316	0.0172	0.0316	0.0422	0.0091	0.0236	0.0087	0.0000							
24	0.0378	0.0240	0.0378	0.0468	0.0318	0.0463	0.0378	0.0463	0.0000						
25	0.0281	0.0170	0.0281	0.0378	0.0255	0.0374	0.0281	0.0374	0.0228	0.0000					
26	0.0374	0.0255	0.0374	0.0478	0.0180	0.0299	0.0374	0.0299	0.0306	0.0081	0.0000				
27	0.0249	0.0146	0.0249	0.0338	0.0234	0.0343	0.0249	0.0343	0.0203	0.0157	0.0245	0.0000			
28	0.0418	0.0075	0.0161	0.0250	0.0164	0.0256	0.0161	0.0256	0.0301	0.0240	0.0334	0.0072	0.0000		
29	0.0265	0.0156	0.0265	0.0360	0.0254	0.0370	0.0265	0.0370	0.0215	0.0167	0.0265	0.0152	0.0234	0.0000	
30	0.0445	0.0080	0.0172	0.0268	0.0180	0.0279	0.0172	0.0279	0.0318	0.0255	0.0361	0.0234	0.0164	0.0081	0.0000
31	0.0370	0.0254	0.0370	0.0473	0.0386	0.0512	0.0370	0.0512	0.0304	0.0265	0.0399	0.0256	0.0344	0.0080	0.0167

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